PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:	WO 97/23614
C12N 15/12, C07K 14/715, C12N 5/10, A01K 67/027, C07K 19/00, C12N 15/62, C07K 16/28, 1/107, C12Q 1/68, G01N 33/50, 33/566, A61K 38/17, 48/00, C12N 1/21 // (C12N 1/21, C12R 1:19)	A1	(43) International Publication Date:	3 July 1997 (03.07.97)

(21) International Application Number: PCT/US96/20621

(22) International Filing Date: 20 December 1996 (20.12.96)

(30) Priority Data:
08/577,788   22 December 1995 (22.12.95)   US
08/706,945   3 September 1996 (03.09.96)   US

(60) Parent Application or Grant
(63) Related by Continuation
US   08/706,945 (CIP)
Filed on   3 September 1996 (03.09.96)

(71) Applicant (for all designated States except US): AMGEN INC. [US/US]; Amgen Center, 1840 De Havilland Drive, Thousand Oaks, CA 91320-1789 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): BOYLE, William, J. [US/US]; 11678 Chestnut Ridge Street, Moorpark, CA (US). LACEY, David, L. [US/US]; 614 Paseo Vista, Thousand Oaks, CA (US). CALZONE, Frank, J. [US/US]; 841 Rim

Crest Circle, Westlake Village, CA (US). CHANG, Ming-Shi [US/US]; 736 Calle Las Colinas, Newbury Park, CA (US).

(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 De Havilland Drive, Thousand Oaks, CA 91320-1789 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

---

(54) Title: OSTEOPROTEGERIN

(57) Abstract

The present invention discloses a secreted polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily and is involved in the regulation of bone metabolism. Also disclosed are nucleic acids encoding osteoprotegerin, polypeptides, recombinant vectors and host cells for expression, antibodies which bind OPG, and pharmaceutical compositions. The polypeptides are used to treat bone diseases characterized by increased resorption such as osteoporosis.

OSTEOPROTEGERIN

Field of the Invention

The invention relates generally to
5   polypeptides involved in the regulation of bone
metabolism. More particularly, the invention relates to
a novel polypeptide, termed osteoprotegerin, which is a
member of the tumor necrosis factor receptor
superfamily. The polypeptide is used to treat bone
10 diseases characterized by increased bone loss such as
osteoporosis.

Background of the Invention

Polypeptide growth factors and cytokines are
15 secreted factors which signal a wide variety of changes
in cell growth, differentiation, and metabolism, by
specifically binding to discrete, surface bound
receptors. As a class of proteins, receptors vary in
their structure and mode of signal transduction. They
20 are characterized by having an extracellular domain that
is involved in ligand binding, and cytoplasmic domain
which transmits an appropriate intracellular signal.
Receptor expression patterns ultimately determine which
cells will respond to a given ligand, while the
25 structure of a given receptor dictates the cellular
response induced by ligand binding. Receptors have been
shown to transmit intracellular signals via their
cytoplasmic domains by activating protein tyrosine, or
protein serine/threonine phosphorylation (e.g., platelet
30 derived growth factor receptor (PDGFR) or transforming
growth factor-β receptor-I (TGFβR-I), by stimulating
G-protein activation (e.g., β-adrenergic receptor), and
by modulating associations with cytoplasmic signal

- 2 -

transducing proteins (e.g., TNFR-1 and Fas/APO) (Heldin, Cell 80, 213-223 (1995)).

The tumor necrosis factor receptor (TNFR) superfamily is a group of type I transmembrane proteins which share a conserved cysteine-rich motif which is repeated three to six times in the extracellular domain (Smith, et al. Cell 76, 953-962 (1994)). Collectively, these repeat units form the ligand binding domains of these receptors (Chen et al., Chemistry 270, 2874-2878 (1995)). The ligands for these receptors are a structurally related group of proteins homologous to TNFα. (Goeddel et al. Cold Spring Harbor Symp. Quart. Biol. 51, 597-609 (1986); Nagata et al. Science 267, 1449-1456 (1995)). TNFα binds to distinct, but closely related receptors, TNFR-1 and TNFR-2. TNFα produces a variety of biological responses in receptor bearing cells, including, proliferation, differentiation, and cytotoxicity and apoptosis (Beutler et al. Ann. Rev. Biochem. 57, 505-518 (1988)).

TNFα is believed to mediate acute and chronic inflammatory responses (Beutler et al. Ann. Rev. Biochem. 57, 505-508 (1988)). Systemic delivery of TNFα induces toxic shock and widespread tissue necrosis. Because of this, TNFα may be responsible for the severe morbidity and mortality associated with a variety of infectious diseases, including sepsis. Mutations in FasL, the ligand for the TNFR-related receptor Fas/APO (Suda et al. Cell 75, 1169-1178 (1993)), is associated with autoimmunity (Fisher et al. Cell 81, 935-946 (1995)), while overproduction of FasL may be implicated in drug-induced hepatitis. Thus, ligands to the various TNFR-related proteins often mediate the serious effects of many disease states, which suggests that agents that

- 3 -

neutralize the activity of these ligands would have
therapeutic value. Soluble TNFR-1 receptors, and
antibodies that bind TNFα, have been tested for their
ability to neutralize systemic TNFα (Loetscher et al.
5   Cancer Cells 3(6), 221-226 (1991)). A naturally
occurring form of a secreted TNFR-1 mRNA was recently
cloned, and its product tested for its ability to
neutralize TNFα activity in vitro and in vivo (Kohno et
al. PNAS USA 87, 8331-8335 (1990)). The ability of this
10   protein to neutralize TNFα suggests that soluble TNF
receptors function to bind and clear TNF thereby
blocking the cytotoxic effects on TNFR- bearing cells.
         An object of the invention to identify new
members of the TNFR super family. It is anticipated
15   that new family members may be transmembrane proteins or
soluble forms thereof comprising extracellular domains
and lacking transmembrane and cytoplasmic domains. We
have identified a new member of the TNFR superfamily
which encodes a secreted protein that is closely related
20   to TNFR-2. By analogy to soluble TNFR-1, the TNFR-2
related protein may negatively regulate the activity of
its ligand, and thus may be useful in the treatment of
certain human diseases.

25   Summary of the Invention

         A novel member of the tumor necrosis factor
receptor (TNFR) superfamily has been identified from a
fetal rat intestinal cDNA library. A full-length cDNA
clone was obtained and sequenced. Expression of the rat
30   cDNA in a transgenic mouse revealed a marked increase in
bones density, particularly in long bones, pelvic bone
and vertebrae. The polypeptide encoded by the cDNA is
termed Osteprotegerin (OPG) and plays a role in
promoting bone accumulation.

- 4 -

The invention provides for nucleic acids
encoding a polypeptide having at least one of the
biological activities of OPG. Nucleic acids which
hybridize to nucleic acids encoding mouse, rat or human
5   OPG as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B
(SEQ ID NO: 122), and 9C-9D (SEQ ID NO: 124) are also
provided. Preferably, OPG is mammalian OPG and more
preferably is human OPG. Recombinant vectors and host
cells expressing OPG are also encompassed as are methods
10 of producing recombinant OPG. Antibodies or fragments
thereof which specifically bind the polypeptide are also
disclosed.

Methods of treating bone diseases are also
provided by the invention. The polypeptides are useful
15 for preventing bone resorption and may be used to treat
any condition resulting in bone loss such as
osteoporosis, hypercalcemia, Paget's disease of bone,
and bone loss due to rheumatoid arthritis or
osteomyelitis, and the like. Bone diseases may also be
20 treated with anti-sense or gene therapy using nucleic
acids of the invention. Pharmaceutical compositions
comprising OPG nucleic acids and polypeptides are also
encompassed.

25 <u>Description of the Figures</u>

Figure 1. A. FASTA analysis of novel EST LORF. Shown
is the deduced FRI-1 amino acid sequence aligned to the
human TNFR-2 sequence. B. Profile analysis of the
30 novel EST LORF shown is the deduced FRI-1 amino acid
sequence aligned to the TNFR-profile. C. Structural
view of TNFR superfamily indicating region which is
homologous to the novel FRI-1.

Figure 2.   Structure and sequence of full length rat OPG
gene, a novel member of the TNFR superfamily.   A.   Map
of pMOB-B1.1 insert.   Box indicates position of LORF
within the cDNA sequence (bold line).   Black box
5   indicates signal peptide, and gray ellipses indicate
position of cysteine-rich repeat sequences.   B, C.
Nucleic acid and protein sequence of the Rat OPG cDNA.
The predicted signal peptide is underlined, and
potential sites of N-linked glycosylation are indicated
10   in bold, underlined letters.   D, E.   Pileup sequence
comparison (Wisconsin GCG Package, Version 8.1) of OPG
with other members of the TNFR superfamily, fas (SEQ ID
NO:128); tnfr1 (SEQ ID NO: 129); sfu-t2 (SEQ ID NO:130);
tnfr2 (SEQ ID NO:131); cd40 (SEQ ID NO:132); osteo (SEQ
15   ID NO:133); ngfr (SEQ ID NO:134); ox40 (SEQ ID NO:135);
41bb (SEQ ID NO:136).

Figure 3.   PepPlot analysis (Wisconsin GCG Package,
Version 8.1) of the predicted rat OPG protein sequence.
20   A. Schematic representation of rat OPG showing
hydrophobic (up) and hydrophilic (down) amino acids.
Also shown are basic (up) and acidic (down) amino acids.
B. Display of amino acid residues that are beta-sheet
forming (up) and beta-sheet breaking down) as defined by
25   Chou and Fasman (Adv. Enz. 47, 45-147 (1948)).   C.
Display of propensity measures for alpha-helix and beta-
sheet (Chou and Fasman, ibid).   Curves above 1.00 show
propensity for alpha-helix or beta-sheet structure.
Structure may terminate in regions of protein where
30   curves drop below 1.00. D. Display of residues that are
alpha-forming (up) or alpha-breaking (down).   E. Display
of portions of the protein sequence that resemble
sequences typically found at the amino end of alpha and
beta structures (Chou and Fasman, ibid).   F. Display of

- 6 -

portions of the protein sequence that resemble sequences
typically found at the carboxyl end of alpha and beta
structures (Chou and Fasman, ibid). G. Display of
portions of the proteins sequence typically found in
5   turns (Chou and Fasman, ibid) H. Display of the helical
hydrophobic moment (Eisenberg et al. Proc. Natl. Acad.
Sci. USA 81, 140-144 (1984)) at each position in the
sequence. I. Display of average hydrophathy based upon
Kyte and Doolittle (J. Mol. Biol. 157, 105-132 (1982))
10 and Goldman et al. (reviewed in Ann. Rev. Biophys.
Biophys. Chem. 15, 321-353 (1986)).

Figure 4. mRNA expression patterns for the OPG cDNA in
human tissues. Northern blots were probed with a 32P-
15 labeled rat cDNA insert (A, left two panels), or with
the human cDNA insert (B, right panel).

Figure 5. Creation of transgenic mice expressing the
OPG cDNA in hepatocytes. Northern blot expression of
20 HE-OPG transgene in mouse liver.

Figure 6. Increase in bone density in OPG transgenic
mice. Panel A-F. Control Mice. G-J, OPG expressing
mice. At necropsy, all animals were radiographed and
25 photographs prepared. In A-F, the radiographs of the
control animals and the one transgenic non-expressor
(#28) are shown. Note that the bones have a clearly
defined cortex and a lucent central marrow cavity. In
contrast, the OPG (G-J) animals have a poorly defined
30 cortex and increased density in the marrow zone.

Figure 7. Increase in trabecular bone in OPG transgenic
mice. A-D. Representative photomicrographs of bones
from control animals. In A and B, low (4X, 10X) power

- 7 -

images of the femurs are shown (Masson Trichrome stain).
Stains for tartrate resistant acid phosphatase (TRAP)
demonstrate osteoclasts (see arrows) both resorbing
cartilage (C) and trabecular bone (D). Note the
5   flattened appearance of osteoclasts on trabecular bone.
E-H.   Representative photomicrographs of bones from
OPG-expressing animals. In E and F, low (4X, 10X) power
images of the femurs are shown (Masson Trichrome stain).
The clear region is the growth plate cartilage, blue
10   stained area is bone, and the red area is marrow. Note
that in contrast to the controls, the trabecular bone
has not been resorbed resulting in the absence of the
usual marrow cavity. Also, the resulting trabeculae have
a variegated appearance with blue and clear areas. The
15   clear areas are remnants of growth plate cartilage that
have never been remodelled. Based on TRAP stains, these
animals do have osteoclasts (see arrows) at the growth
plate (G), which may be reduced in number. However, the
surfaces of the trabeculae away from the growth plate
20   are virtually devoid of osteoclasts (H), a finding that
stands in direct contrast with the control animals (see
D).

Figure 8. HE-OPG expressors do not have a defect in
25   monocyte-macrophage development. One cause for
osteopetrosis in mice is defective M-CSF production due
to a point mutation in the M-CSF gene. This results in
a marked deficit of circulating and tissue based
macrophages. The peripheral blood of OPG expressors
30   contained monocytes as assessed by H1E analysis. To
affirm the presence of tissue macrophages,
immnohistochemistry was performed using F480 antibodies,
which recognize a cell surface antigen on murine
macrophages. A and C show low power (4X)

- 8 -

photomicrographs of the spleens from normal and CR1
overexpressors. Note that both animals have numerous
F480 positive cells. Monocyte-macrophages were also
present in the marrow of normal (B) and HE-OPG
5 overexpressors (D) (40X).

Figure 9. Structure and sequence of mouse and human OPG
cDNA clones. A, B. Mouse cDNA and protein sequence.
C, D. Human cDNA and protein sequence. The predicted
10 signal peptides are underlined, and potential sites of
N-linked glycosylation are indicated in bold. E, F.
Sequence alignment and comparison of rat, mouse and
human OPG amino acid sequences.

15 Figure 10. Comparison of conserved sequences in
extracellular domain of TNFR-1 and human OPG.
PrettyPlot (Wisconsin GCG Package, Version 8.1) of the
TNFR1 and OPG alignment described in example 6. Top
line, human TNFR1 sequences encoding domains 1-4.
20 Bottom line, human OPG sequences encoding domains 1-4.
Conserved residues are highlighted by rectangular boxes.

Figure 11. Three-dimensional representation of human
OPG. Side-view of the Molescript display of the
25 predicted 3-dimensional structure of human OPG residues
25 through 163, (wide line), co-crystallized with human
TNFβ (thin line). As a reference for orientation, the
bold arrows along the OPG polypeptide backbone are
pointing in the N-terminal to C-terminal direction. The
30 location of individual cysteine residue side chains are
inserted along the polypeptide backbone to help
demonstrate the separate cysteine-rich domains. The
TNFβ molecule is aligned as described by Banner et al.
(1993).

Figure 12. Structure of OPG cysteine-rich domains.
Alignment of the human (top line SEQ ID NO:136) and
mouse (bottom line) OPG amino acid sequences
5   highlighting the predicted domain structure of OPG. The
polypeptide is divided into two halves; the N-terminus
(A), and C-terminus (B). The N-terminal half is
predicted to contain four cysteine rich domains (labeled
1-4). The predicted intrachain disulfide bonds are
10   indicated by bold lines, labeled "SS1", "SS2", or "SS3".
Tyrosine 28 and histidine 75 (underlined) are predicted
to form an ionic interaction. Those amino acids
predicted to interact with an OPG ligand are indicated
by bold dots above the appropriate residue. The
15   cysteine residues located in the C-terminal half of OPG
are indicated by rectangular boxes.

Figure 13. Expression and secretion of full length and
truncated mouse OPG-Fc fusion proteins. A. Map
20   indicating points of fusion to the human IgG1 Fc domain
are indicated by arrowheads. B. Silver stain of a SDS-
polyacrylamide gel of conditioned media obtained from
cells expressing either Fl.Fc (Full length OPG fused to
Fc at Leucine 401) or CT.Fc (Carboxy-terminal truncated
25   OPG fused to Fc at threonine 180) fusion protein
expression vectors. Lane 1, parent pCEP4 expression
vector cell line; Lane 2, Fl.Fc vector cell line; Lane
3, CT.Fc vector cell line. C. Western blot of
conditioned media obtained from Fl.Fc and CT.Fc fusion
30   protein expression vectors probed with anti-human IgG1
Fc domain (Pierce). Lane 1, parent pCEP4 expression
vector cell line; Lane 2, Fl.Fc vector cell line; Lane
3, CT.Fc vector cell line.

Figure 14.   Expression of human OPG in E. coli.   A.
Construction of a bacterial expression vector.   The LORF
of the human OPG gene was amplified by PCR, then joined
to a oligonucleotide linker fragment (top strand is SEQ
5   ID NO:137; bottom strand is SEQ ID NO:127), and ligated
into pAMG21 vector DNA.   The resulting vector is capable
of expressing OPG residues 32-401 linked to a N-terminal
methionine residue.   B   SDS-PAGE analysis of uninduced
and induced bacterial harboring the pAMG21-human OPG -
10 32-401 plasmid.   Lane 1, MW standards; lane 2, uninduced
bacteria; lane 3, 30°C induction; lane 4, 37°C
induction; lane 5, whole cell lysate from 37°C
induction; lane 6, soluble fraction of whole cell
lysate; lane 7, insoluble fraction of whole cell lysate;
15 lane 8, purified inclusion bodies obtained from whole
cell lysate.

Figure 15.   Analysis of recombinant murine OPG produced
in CHO cells by SDS-PAGE and western blotting.   An equal
20 amount of CHO conditioned media was applied to each lane
shown, and was prepared by treatment with either
reducing sample buffer (left lane), or non-reducing
sample buffer (right lane).   After electrophoresis, the
resolved proteins were transferred to a nylon membrane,
25 then probed with anti-OPG antibodies.   The relative
positions of the 55 kd monomeric and 100 kd dimeric
forms of OPG are indicated by arrowheads.

Figure 16.   Pulse-chase analysis of recombinant murine
30 OPG produced in CHO cells.   CHO cells were pulse-labeled
with ^{35}S-methionine/cysteine, then chased for the
indicated time.   Metabolically labeled cultures were
separated into both conditioned media and cells, and
detergent extracts were prepared from each, clarified,

- 11 -

then immunoprecipitated with anti-OPG antibodies. The
immunoprecipitates were the resolved by SDS-PAGE, and
exposed to film. Top left and right panels; samples
analyzed under non-reducing conditions. Lower left and
5   right panels; samples analyzed under reducing
conditions. Top and bottom left panels; Cell extracts.
Top and bottom right panels; Conditioned media extracts.
The relative mobility of the 55 kd monomeric and 100 kd
dimeric forms of OPG are indicated by arrowheads.
10

Figure 17. Expression of OPG in the CTLL-2 cell line.
Serum-free conditioned media from CTLL-2 cells and CHO-
mu OPG [1-401] transfected cells was prepared,
concentrated, then analyzed by non-reducing SDS-PAGE and
15 western blotting. Left lane; CTLL-2 conditioned media.
Right lane; CHO-muOPG conditioned media. The relative
mobility of the 55 kd monomeric and 100 kd dimeric forms
of OPG are indicated by arrowheads.

20 Figure 18. Detection of OPG expression in serum samples
and liver extracts obtained from control and OPG
transgenic mice. Transgenic mice were constructed as
described in Example 4. OPG expression was visualized
after SDS-PAGE followed by Western blotting using
25 anti-OPG antibodies.

Figure 19. Effects of huOPG [22-401]-Fc fusion protein
on osteoclast formation in vitro. The osteoclast
forming assay was performed as described in Example 11A
30 in the absence (control) or presence of the indicated
amounts of huOPG [22-401]-Fc fusion. Osteoclast
formation was visualized by histochemical staining for
tartrate acid phosphatase (TRAP).). A. OPG added to
100 ng/ml. D. OPG added to 0.1 ng/ml. E. OPG added to

- 12 -

0.01 ng/ml.   F.   OPG added to 0.001 ng/ml.   G.   Control.
No OPG added.

Figure 20.   Decrease in osteoclast culture TRAP activity
5   with increasing amounts of OPG.   Indicated
concentrations of huOPG [22-401]-Fc fusion protein were
added to osteoclast forming assay and TRAP activity
quantitated as described in Example 11A.

10   Figure 21.   Effect of OPG on a terminal stage of
osteoclast differentiation.   huOPG [22-401]-Fc fusion
was added to the osteoclast forming assay during the
intermediate stage of osteoclast maturation (days 5-6;
OPG-CTL) or during the terminal stage of osteoclast
15   maturation (days 7-15; CTL-OPG).   TRAP activity was
quantitated and compared with the activity observed in
the absence of OPG (CTL-CTL) in the presence of OPG
throughout (OPG-OPG).

20   Figure 22.   Effects of IL-1β, IL-1α and OPG on blood
ionized calcium in mice.   Levels of blood ionized
calcium were monitored after injection of IL-1β alone,
IL-1α alone, IL-1β plus muOPG [22-401]-Fc, IL-1α plus
MuOPG [22-401]-Fc, and muOPG [22-401]-Fc alone.   Control
25   mice received injections of phosphate buffered saline
(PBS) only.   IL-1B experiment shown in A; IL-1α
experiment shown in B.

Figure 23.   Effects of OPG on calvarial osteoclasts in
30   control and IL1-treated mice.   Histological methods for
analyzing mice calvarial bone samples are described in
Example 11B.   Arrows indicate osteoclasts present in day
2-treated mice.   Calvarial samples of mice receiving
four PBS injections daily (A), one injection of IL-1 and

- 13 -

three injections of PBS daily (B), one injection of PBS
and three injections of OPG daily (C), one injection of
IL-1 and three injections of OPG daily.

5   Figure 24. Radiographic analysis of bone accumulation
in marrow cavity of normal mice. Mice were injected
subcutaneously with saline (A) or muOPG [22-401]-Fc
fusion (5mg/kg/d) for 14 days (B) and bone density
determined as described in Example 11C.

10

Figure 25. Histomorphometric analysis of bone
accumulation in marrow cavity of normal mice. Injection
experiments and bone histology performed as described in
Example 11C.

15

Figure 26. Histology analysis of bone accumulation in
marrow cavity of normal mice. Injection experiments and
bone histology performed as described in Example 11C.
A. Saline injection B. Injection of muOPG [22-401]-Fc
20 fusion.

Figure 27. Activity of OPG administered to
ovariectomized rats. In this two week experiment the
trend to reduced bone density appears to be blocked by
25 OPG or other anti-resorptive therapies. DEXA
measurements were taken at time of ovariectomy and at
week 1 and week 2 of treatment. The results are
expressed as % change from the initial bone density
(Mean +/- SEM).

30

Figure 28. Bone density in the femoral metaphysis,
measured by histomorphometric methods, tends to be lower
in ovariectomized rats (OVX) than sham operated animals
(SHAM) 17 days following ovariectomy. This effect was

- 14 -

blocked by OPG-Fc, with OPG-Fc treated ovariectomized rats (OVX+OPG) having significantly higher bone density than vehicle treated ovariectomized rats (OVX). (Mean +/- SEM).

Detailed Description of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily was identified as an expressed sequence tag (EST) isolated from a fetal rat intestinal cDNA library . The structures of the full-length rat cDNA clones and the corresponding mouse and human cDNA clones were determined as described in Examples 1 and 6. The rat, mouse and human genes are shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), respectively. All three sequences showed strong similarity to the extracellular domains of TNFR family members. None of the full-length cDNA clones isolated encoded transmembrane and cytoplasmic domains that would be expected for membrane-bound receptors, suggesting that these cDNAs encode soluble, secreted proteins rather than cell surface receptors. A portion of the human gene spanning nucleotides 1200-1353 shown in Figure 9D was deposited in the Genebank database on November 22, 1995 under accession no. 17188769.

The tissue distribution of the rat and human mRNA was determined as described in Example 2. In rat, mRNA expression was detected in kidney, liver, placenta and heart with the highest expression in the kidney. Expression in skeletal muscle and pancreas was also detected. In humans, expression was detected in the same tissues along with lymph node, thymus, spleen and appendix.

- 15 -

The rat cDNA was expressed in transgenic mice (Example 3) using the liver-specific ApoE promoter expression system. Analysis of expressors showed a marked increase in bone density, particularly in long
5 bones (femurs), vertebrae and flat bones (pelvis). Histological analysis of stained sections of bone showed severe osteopetrosis (see Example 4) indicating a marked imbalance between bone formation and resorption which has led to a marked accumulation of bone and cartilage.
10 A decrease in the number of trabecular osteoclasts in the bones of OPG expressor animals indicate that a significant portion of the activity of the TNFR-related protein may be to prevent bone resorption, a process mediated by osteoclasts. In view of the activity in
15 transgenic expressors, the TNFR-related proteins described herein are termed OPGs.

Using the rat cDNA sequence, mouse and human cDNA clones were isolated (Example 5). Expression of mouse OPG in 293 cells and human OPG in E. coli is
20 described in Examples 7 and 8. Mouse OPG was produced as an Fc fusion which was purified by Protein A affinity chromatography. Also described in Example 7 is the expression of full-length and truncated human and mouse OPG polypeptides in CHO and 293 cells either as fusion
25 polypeptides to the Fc region of human IgG1 or as unfused polypeptides. The expression of full-length and truncated human and mouse OPGs in E. coli either as Fc fusion polypeptides or as unfused polypeptides is described in Example 8. Purification of recombinantly
30 produced mammalian and bacterial OPG is described in Example 10.

The biological activity of OPG was determined using an in vitro osteoclast maturation assay, an in vivo model of interleukin-1 (IL-1) induced

- 16 -

hypercalcemia, and injection studies of bone density in normal mice (see Example 11). The following OPG recombinant proteins produced in CHO or 293 cells demonstrated activity in the in E. coli osteoclast
5   maturation assay: muOPG [22-185]-Fc, muOPG [22-194]-Fc, muOPG [22-401]Fc, muOPG [22-401], huOPG [22-201]-Fc, huOPG [22-401]-Fc. muOPG [22-180]-Fc produced in CHO cells and huOPG met[32-401] produced in E. coli did not demonstrate activity in the in vitro assay.
10   OPG from several sources was produced as a dimer and to some extent as a higher multimer. Rat OPG [22-401] produced in transgenic mice, muOPG [22-401] and huOPG [22-401] produced as a recombinant polypeptide in CHO cells, and OPG expressed as a naturally occurring
15   product from a cytotoxic T cell line were predominantly dimers and trimers when analyzed on nonreducing SDS gels (see Example 9). Truncated OPG polypeptides having deletions in the region of amino acids 186-401 (e.g., OPG [1-185] and OPG [1-194]) were predominantly
20   monomeric suggesting that the region 186-401 may be involved in self-association of OPG polypeptides. However, huOPG met[32-401] produced in E. coli was largely monomeric.
            OPG may be important in regulating bone
25   resorption. The protein appears to act as a soluble receptor of the TNF family and may prevent a receptor-ligand interaction involved in the osteolytic pathway. One aspect of the regulation appears to be a reduction in the number of osteoclasts.
30

Nucleic Acids

            The invention provides for an isolated nucleic acid encoding a polypeptide having at least one of the biological activities of OPG. As described herein, the

- 17 -

biological activities of OPG include, but are not
limited to, any activity involving bone metabolism and
in particular, include increasing bone density. The
nucleic acids of the invention are selected from the
5   following:

a) the nucleic acid sequences as shown in Figures
2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D
(SEQ ID NO:124) or complementary strands thereof;

b) the nucleic acids which hybridize under
10 stringent conditions with the polypeptide-encoding
region in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID
NO:122), and 9C-9D (SEQ ID NO:124); and

c) nucleic acids which hybridize under stringent
conditions with nucleotides 148 through 337 inclusive as
15 shown in Figure 1A.

d) the nucleic acid sequences which are degenerate
to the sequences in (a) and (b).

The invention provides for nucleic acids which
encode rat, mouse and human OPG as well as nucleic acid
20 sequences hybridizing thereto which encode a polypeptide
having at least one of the biological activities of OPG.
Also provided for are nucleic acids which hybridize to a
rat OPG EST encompassing nucleotides 148-337 as shown in
Figure 1A. The conditions for hybridization are
25 generally of high stringency such as 5xSSC, 50%
formamide and 42°C described in Example 1 of the
specification. Equivalent stringency to these
conditions may be readily obtained by adjusting salt and
organic solvent concentrations and temperature. The
30 nucleic acids in (b) encompass sequences encoding OPG-
related polypeptides which do not undergo detectable
hybridization with other known members of the TNF
receptor superfamily. In a preferred embodiment, the
nucleic acids are as shown in Figures 2B-2C (SEQ ID

- 18 -

NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID
NO:124).

      The length of hybridizing nucleic acids of the
invention may be variable since hybridization may occur
5   in part or all of the polypeptide-encoding regions as
shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID
NO:122), and 9C-9D (SEQ ID NO:124), and may also occur
in adjacent noncoding regions. Therefore, hybridizing
nucleic acids may be truncations or extensions of the
10   sequences shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B
(SEQ ID NO:122), and 9C-9D (SEQ ID NO:124). Truncated
or extended nucleic acids are encompassed by the
invention provided they retain one or more of the
biological properties of OPG. The hybridizing nucleic
15   acids may also include adjacent noncoding regions which
are 5' and/or 3' to the OPG coding region. The
noncoding regions include regulatory regions involved in
OPG expression, such as promoters, enhance,
translational initiation sites, transcription
20   termination sites and the like.

      Hybridization conditions for nucleic acids are
described in Sambrook et al. <u>Molecular Cloning: A
Laboratory Manual</u>, 2nd ed. Cold Spring Harbor Laboratory
Press, Cold Spring Harbor, New York (1989)

25   DNA encoding rat OPG was provided in plasmid
pMO-B1.1 deposited with the American Type Culture
Collection, Rockville, MD on December 27, 1995 under
ATCC accession no. 69970. DNA encoding mouse OPG was
provided in plasmid pRcCMV-murine OPG deposited with the
30   American Type Culture Collection, Rockville, MD on
December 27, 1995 under accession no. 69971. DNA
encoding human OPG was provided in plasmid pRcCMV -
human OPG deposited with the American Type Culture
Collection, Rockville, MD on December 27, 1995 under

- 19 -

accession no. 69969. The nucleic acids of the invention
will hybridize under stringent conditions to the DNA
inserts of ATCC accession nos. 69969, 69970, and 69971
and have at least one of the biological activities of
OPG.

     Also provided by the invention are derivatives
of the nucleic acid sequences as shown in Figures 2B, 9A
and 9B. As used herein, derivatives include nucleic
acid sequences having addition, substitution, insertion
or deletion of one or more residues such that the
resulting sequences encode polypeptides having one or
more amino acid residues which have been added, deleted,
inserted or substituted and the resulting polypeptide
has the activity of OPG. The nucleic acid derivatives
may be naturally occurring, such as by splice variation
or polymorphism, or may be constructed using site-
directed mutagenesis techniques available to the skilled
worker. One example of a naturally occurring variant of
OPG is a nucleic acid encoding a lys to asn change at
residue 3 within the leader sequence (see Example 5).
It is anticipated that nucleic acid derivatives will
encode amino acid changes in regions of the molecule
which are least likely to disrupt biological activity.
Other derivatives include a nucleic acid encoding a
membrane-bound form of OPG having an extracellular
domain as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B
(SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) along with
transmembrane and cytoplasmic domains.

     In one embodiment, derivatives of OPG include
nucleic acids encoding truncated forms of OPG having one
or more amino acids deleted from the carboxy terminus.
Nucleic acids encoding OPG may have from 1 to 216 amino
acids deleted from the carboxy terminus. Optionally, an
antibody Fc region may extend from the new carboxy

- 20 -

terminus to yield a biologically active OPG-Fc fusion
polypeptide. (see Example 11). In preferred
embodiments, nucleic acids encode OPG having the amino
acid sequence from residues 22-185, 22-189, 22-194 or

5   22-201 (using numbering in Figure 9E-F) and optionally,
encoding an Fc region of human IgG.

Also included are nucleic acids encoding
truncated forms of OPG having one or more amino acids
deleted from the amino terminus. Truncated forms

10   include those lacking part or all the 21 amino acids
comprising the leader sequence. Additionally, the
invention provides for nucleic acids encoding OPG having
from 1 to 10 amino acids deleted from the mature amino
terminus (at residue 22) and ,optionally, having from 1

15   to 216 amino acids deleted from the carboxy terminus (at
residue 401). Optionally, the nucleic acids may encode
a methionine residue at the amino terminus. Examples of
such OPG truncated polypeptides are described in Example
8.

20   Examples of the nucleic acids of the invention
include cDNA, genomic DNA, synthetic DNA and RNA. cDNA
is obtained from libraries prepared from mRNA isolated
from various tissues expressing OPG. In humans, tissue
sources for OPG include kidney, liver, placenta and

25   heart. Genomic DNA encoding OPG is obtained from
genomic libraries which are commercially available from
a variety of species. Synthetic DNA is obtained by
chemical synthesis of overlapping oligonucleotide
fragments followed by assembly of the fragments to

30   reconstitute part or all of the coding region and
flanking sequences (see U.S. Patent No. 4,695,623
describing the chemical synthesis of interferon genes).
RNA is obtained most easily by procaryotic expression

- 22 -

secreted polypeptides which may be recovered from the
growth medium.

Vectors for the expression of OPG contain at a
minimum sequences required for vector propogation and
5   for expression of the cloned insert. These sequences
include a replication origin, selection marker,
promoter, ribosome binding site, enhancer sequences, RNA
splice sites and transcription termination site.
Vectors suitable for expression in the aforementioned
10   host cells are readily available and the nucleic acids
of the invention are inserted into the vectors using
standard recombinant DNA techniques. Vectors for
tissue-specific expression of OPG are also included.
Such vectors include promoters which function
15   specifically in liver, kidney or other organs for
production in mice, and viral vectors for the expression
of OPG in targeted human cells.

Using an appropriate host-vector system, OPG
is produced recombinantly by culturing a host cell
20   transformed with an expression vector containing nucleic
acid sequences encoding OPG under conditions such that
OPG is produced, and isolating the product of
expression. OPG is produced in the supernatant of
transfected mammalian cells or in inclusion bodies of
25   transformed bacterial host cells. OPG so produced may
be purified by procedures known to one skilled in the
art as described below. The expression of OPG in
mammalian and bacterial host systems is described in
Examples 7 and 8. Expression vectors for mammalian
30   hosts are exemplified by plasmids such as pDSRα
described in PCT Application No. 90/14363. Expression
vectors for bacterial host cells are exemplified by
plasmids pAMG21 and pAMG22-His described in Example 8.
Plasmid pAMG21 was deposited with the American Type

- 21 -

vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

5   Nucleic acid sequences of the invention are used for the detection of OPG sequences in biological samples in order to determine which cells and tissues are expressing OPG mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to OPG. Such screening is well within the capabilities of one skilled in the art using appropriate

10   hybridization conditions to detect homologus sequences. The nucleic acids are also useful for modulating the expression of OPG levels by anti-sense therapy or gene therapy. The nucleic acids are also used for the development of transgenic animals which may be used for

15   the production of the polypeptide and for the study of biological activity (see Example 3).

Vectors and Host Cells

Expression vectors containing nucleic acid

20   sequences encoding OPG, host cells transformed with said vectors and methods for the production of OPG are also provided by the invention. An overview of expression of recombinant proteins is found in Methods of Enzymology v. 185, Goeddel, D.V. ed. Academic Press (1990).

25   Host cells for the production of OPG include procaryotic host cells, such as E. coli, yeast, plant, insect and mammalian host cells. E. coli strains such as HB101 or JM101 are suitable for expression. Preferred mammalian host cells include COS, CHOd-, 293,

30   CV-1, 3T3, baby hamster kidney (BHK) cells and others. Mammalian host cells are preferred when post-translational modifications, such as glycosylation and polypeptide processing, are important for OPG activity. Mammalian expression allows for the production of

- 23 -

Culture Collection, Rockville, MD on July 24, 1996 under
accession no. 98113. Plasmid pAMG22-His was deposited
with the American Type Culture Collection, Rockville, MD
on July 24, 1996 under accession no. 98112. It is

5   anticipated that the specific plasmids and host cells
described are for illustrative purposes and that other
available plasmids and host cells could also be used to
express the polypeptides.

The invention also provides for expression of

10   OPG from endogenous nucleic acids by _in vivo_ or _ex vivo_
recombination events to allow modulation of OPG from the
host chromosome. Expression of OPG by the introduction
of exogenous regulatory sequences (e.g. promoters or
enhancers) capable of directing the production of OPG

15   from endogenous OPG coding regions is also encompassed.
Stimulation of endogenous regulatory sequences capable
of directing OPG production (e.g. by exposure to
transcriptional enhancing factors) is also provided by
the invention.

20

Polypeptides

The invention provides for OPG, a novel member
of the TNF receptor superfamily, having an activity
associated with bone metabolism and in particular having

25   the activity of inhibiting bone resorption thereby
increasing bone density. OPG refers to a polypeptide
having an amino acid sequence of mouse, rat or human OPG
or a derivative thereof having at least one of the
biological activities of OPG. The amino acid sequences

30   of rat, mouse and human OPG are shown in Figures 2B-2C
(SEQ ID NO:121), 9A-9B (SEQ ID NO:123), and 9C-9D (SEQ
ID NO:125) respectively. A derivative of OPG refers to
a polypeptide having an addition, deletion, insertion or
substitution of one or more amino acids such that the

- 24 -

resulting polypeptide has at least one of the biological activities of OPG. The biological activities of OPG include, but are not limited to, activities involving bone metabolism. Preferably, the polypeptides will have
5   the amino terminal leader sequence of 21 amino acids removed.

OPG polypeptides encompassed by the invention include rat [1-401], rat [22-180], rat [22-401], rat [22-401]-Fc fusion, rat [1-180]-Fc fusion, mouse [1-
10   401], mouse [1-180], mouse [22-401], human [1-401], mouse [22-180], human [22-401], human [22-180], human [1-180], human [22-180]-Fc fusion and human met-32-401. Amino acid numbering is as shown in SEQ ID NO:121 (rat), SEQ ID NO:123 (mouse) and SEQ ID NO:125 (human). Also
15   encompassed are polypeptide derivatives having deletions or carboxy-terminal truncations of part or all of amino acids residues 180-401 of OPG; one or more amino acid changes in residues 180-401; deletion of part or all of a cysteine-rich domain of OPG, in particular deletion of
20   the distal (carboxy-terminal) cysteine-rich domain; and one or more amino acid changes in a cysteine-rich domain, in particular in the distal (carboxy-terminal) cysteine-rich domain. In one embodiment, OPG has from 1 to about 216 amino acids deleted from the carboxy
25   terminus. In another embodiment, OPG has from 1 to about 10 amino acids deleted from the mature amino terminus (wherein the mature amino terminus is at residue 22) and, optionally, has from 1 to about 216 amino acids deleted from the carboxy terminus.
30   Additional OPG polypeptides encompassed by the invention include the following: human [22-180]-Fc fusion, human [22-201]-Fc fusion, human [22-401]-Fc fusion, mouse [22-185]-Fc fusion, mouse [22-194]-Fc fusion. These polypeptides are produced in mammalian

- 25 -

host cells, such as CHO or 293 cells, Additional OPG
polypeptides encompassed by the invention which are
expressed in procaryotic host cells include the
following: human met[22-401], Fc-human met[22-401]
5   fusion (Fc region is fused at the amino terminus of the
full-length OPG coding sequence as described in Example
8), human met[22-401]-Fc fusion (Fc region fused to the
full-lengh OPG sequence), Fc-mouse met[22-401] fusion,
mouse met[22-401]-Fc fusion, human met[27-401], human
10 met[22-185], human met[22-189], human met[22-194], human
met[22-194] (P25A), human met [22-194] (P26A), human
met[27-185], human met[27-189], human met[27-194], human
met-arg-gly-ser-(his)6 [22-401], human met-lys [22-401],
human met-(lys)3-[22-401], human met[22-401]-Fc (P25A),
15 human met[22-401] (P25A), human met[22-401] (P26A), human
met[22-401] (P26D), mouse met[22-401], mouse met[27-
401], mouse met[32-401], mouse met[27-180], mouse
met[22-189], mouse met[22-194], mouse met[27-189], mouse
met[27-194], mouse met-lys[22-401], mouse HEK[22-
20 401](A45T), mouse met-lys-(his)7[22-401], mouse met-
lys[22-401]-(his)7 and mouse met[27-401] (P33E, G36S,
A45P). It is understood that the above OPG polypeptides
produced in procaryotic host cells have an amino-
terminal methionine residue, if such a residue is not
25 indicated. In specific examples, OPG-Fc fusion were
produced using a 227 amino acid region of human IgG1-γ1
was used having the sequence as shown in Ellison et al.
(Nuc. Acids Res. 10, 4071-4079 (1982)). However,
variants of the Fc region of human IgG may also be used.
30   Analysis of the biological activity of
carboxy-terminal OPG truncations fused to the human IgG1
Fc region indicates a portion of OPG of about 164 amino
acids which is required for activity. This region
encompasses amino acids 22-185, preferably those in

Figure 9C-9D (SEQ ID NO:125), and comprises four cysteine-rich domains characteristic of the cysteine-rich domains of TNFR extraceullular domains.

5   Using the homology between OPG and the extracellular ligand binding domains of TNF receptor family members, a three-dimensional model of OPG was generated based upon the known crystal structure of the extracellular domain of TNFR-I (see Example 6). This model was used to identify those residues within OPG 10 which may be important for biological activity. Cysteine residues that are involved in maintaining the structure of the four cysteine-rich domains were identified. The following disulfide bonds were identified in the model: Domain 1: cys41 to cys54, cys44 15 to cys62, tyr23 and his 66 may act to stabilize the structure of this domain; Domain 2: cys65 to cys80, cys83 to cys98, cys87 to cys105; Domain 3: cys107 to cys118, cys124 to cys142; Domain 4: cys145 to cys160, cys166 to cys185. Residues were also identified which 20 were in close proximity to TNFβ as shown in Figures 11 and 12A-12B. In this model, it is assumed that OPG binds to a corresponding ligand; TNFβ was used as a model ligand to simulate the interaction of OPG with its ligand. Based upon this modeling, the following 25 residues in OPG may be important for ligand binding: glu34, lys43, pro66 to gln91 (in particular, pro66, his68, tyr69, tyr70, thr71, asp72, ser73, his76, ser77, asp78, glu79, leu81, tyr82, pro85, val86, lys88, glu90 and gln91), glu153 and ser155.

30   Alterations in these amino acid residues, either singly or in combination, may alter the biological activity of OPG. For example, changes in specific cysteine residues may alter the structure of individual cysteine-rich domains, whereas changes in

- 27 -

residues important for ligand binding may affect physical interactions of OPG with ligand. Structural models can aid in identifying analogs which have more desirable properties, such as enhanced biological

5   activity, greater stability, or greater ease of formulation.

The invention also provides for an OPG multimer comprising OPG monomers. OPG appears to be active as a multimer (e.g, dimer, trimer of a higher

10   number of monomers). Preferably, OPG multimers are dimers or trimers. OPG multimers may comprise monomers having the amino acid sequence of OPG sufficient to promote multimer formation or may comprise monomers having heterologous sequences such as an antibody Fc

15   region. Analysis of carboxy-terminal deletions of OPG suggest that at least a portion of the region 186-401 is involved in association of OPG polypeptides. Substitution of part or all of the region of OPG amino acids 186-401 with an amino acid sequence capable of

20   self-association is also encompassed by the invention. Alternatively, OPG polypeptides or derivatives thereof may be modified to form dimers or multimers by site directed mutagenesis to create unpaired cysteine residues for interchain disulfide bond romation, by

25   photochemical crosslinking, such as exposure to ultraviolet light, or by chemical crosslinking with bifunctional linker molecules such as bifunctional polyethylene glycol and the like.

Modifications of OPG polypeptides are

30   encompassed by the invention and include post-translational modifications (e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked

- 28 -

or O-linked carbohydrate chains, and addition of an
N-terminal methionine residue as a result of procaryotic
host cell expression. The polypeptides may also be
modified with a detectable label, such as an enzymatic,
5   fluorescent, isotopic or affinity label to allow for
detection and isolation of the protein.

Further modifications of OPG include chimeric
proteins wherein OPG is fused to a heterologous amino
acid sequence. The heterologous sequence may be any
10   sequence which allows the resulting fusion protein to
retain the activity of OPG. The heterologous sequences
include for example, immunoglobulin fusions, such as Fc
fusions, which may aid in purification of the protein.
A heterologous sequence which promotes association of
15   OPG monomers to form dimers, trimers and other higher
multimeric forms is preferred.

The polypeptides of the invention are isolated
and purified from other polypeptides present in tissues,
cell lines and transformed host cells expressing OPG, or
20   purified from components in cell cultures containing the
secreted protein. In one embodiment, the polypeptide is
free from association with other human proteins, such as
the expression product of a bacterial host cell.

Also provided by the invention are chemically
25   modified derivatives of OPG which may provide additional
advantages such as increasing stability and circulating
time of the polypeptide, or decreasing immunogenicity
(see U.S. Patent No. 4,179,337). The chemical moieties
for derivitization may be selected from water soluble
30   polymers such as polyethylene glycol, ethylene
glycol/propylene glycol copolymers,
carboxymethylcellulose, dextran, polyvinyl alcohol and
the like. The polypeptides may be modified at random
positions within the molecule, or at predetermined

- 29 -

positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g. EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20: 1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydrl groups may also be used as a reactive group

for attaching the polyethylene glycol molecule(s).
Preferred for therapeutic purposes is attachment at an
amino group, such as attachment at the N-terminus or
lysine group.

5   One may specifically desire N-terminally
chemically modified protein.   Using polyethylene glycol
as an illustration of the present compositions, one may
select from a variety of polyethylene glycol molecules
(by molecular weight, branching, etc.), the proportion
10 of polyethylene glycol molecules to protein (or peptide)
molecules in the reaction mix, the type of pegylation
reaction to be performed, and the method of obtaining
the selected N-terminally pegylated protein.   The method
of obtaining the N-terminally pegylated preparation
15 (i.e., separating this moiety from other monopegylated
moieties if necessary) may be by purification of the
N-terminally pegylated material from a population of
pegylated protein molecules.   Selective N-terminal
chemically modification may be accomplished by reductive
20 alkylation which exploits differential reactivity of
different types of primary amino groups (lysine versus
the N-terminal) available for derivatization in a
particular protein.   Under the appropriate reaction
conditions, substantially selective derivatization of
25 the protein at the N-terminus with a carbonyl group
containing polymer is achieved.

             Synthetic OPG dimers may be prepared by
various chemical crosslinking procedures.   OPG monomers
may be chemically linked in any fashion that retains or
30 enhances the biological activity of OPG.   A variety of
chemical crosslinkers may be used depending upon which
properties of the protein dimer are desired.   For
example, crosslinkers may be short and relatively rigid
or longer and more flexible, may be biologically

- 31 -

reversible, and may provide reduced immunogenicity or longer pharmacokinetic half-life.

In one example, OPG molecules are linked through the amino terminus by a two step synthesis (see
5   Example 12). In the first step, OPG is chemically modified at the amino terminus to introduce a protected thiol, which after purification is deprotected and used as a point of attachment for site-specific conjugation through a variety of crosslinkers with a second OPG
10 molecule. Amino-terminal crosslinks include, but are not limited to, a disulfide bond, thioether linkages using short-chain, bis-functional aliphatic crosslinkers, and thioether linkages to variable length, bifunctional polyethylene glycol crosslinkers (PEG
15 "dumbbells"). Also encompassed by PEG dumbbell synthesis of OPG dimers is a byproduct of such synthesis, termed a "monobell". An OPG monobell consists of a monomer coupled to a linear bifunctional PEG with a free polymer terminus. Alternatively, OPG
20 may be crosslinked directly through a variety of amine specific homobifunctional crosslinking techniques which include reagents such as: diethylenetriaminepentaacetic dianhydride (DTPA), p-benzoquinone (pBQ) or bis(sulfosuccinimidyl) suberate (BS^3) as well as others
25 known in the art. It is also possible to thiolate OPG directly with reagents such as iminothiolane in the presence of a variety of bifunctional, thiol specific crosslinkers, such as PEG bismaleimide, and achieve dimerization and/or dumbbells in a one step process.
30   A method for the purification of OPG from natural sources and from transfected host cells is also included. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The

- 32 -

chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-OPG antibody or biotin-streptavidin affinity
5   complex and the like.

Antibodies

          Also encompassed by the invention are antibodies specifically binding to OPG. Antigens for
10 the generation of antibodies may be full-length polypeptides or peptides spanning a portion of the OPG sequence. Immunological procedures for the generation of polyclonal or monoclonal antibodies reactive with OPG are known to one skilled in the art (see, for example,
15 Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. (1988)). Antibodies so produced are characterized for binding specificity and epitope recognition using standard enzyme-linked immunosorbent assays. Antibodies
20 also include chimeric antibodies having variable and constant domain regions derived from different species. In one embodiment, the chimeric antibodies are humanized antibodies having murine variable domains and human constant domains. Also encompassed are complementary
25 determining regions grafted to a human framework (so-called CDR-grafted antibodies). Chimeric and CDR-grafted antibodies are made by recombinant methods known to one skilled in the art. Also encompassed are human antibodies made in mice.
30   Anti-OPG antibodies of the invention may be used as an affinity reagent to purify OPG from biological samples (see Example 10). In one method, the antibody is immobilized on CnBr-activated Sepharose and a column of antibody-Sepharose conjugate is used to

- 33 -

remove OPG from liquid samples. Antibodies are also
used as diagnostic reagents to detect and quantitate OPG
in biological samples by methods described below.

5   <u>Pharmaceutical compositions</u>

         The invention also provides for pharmaceutical
compositions comprising a therapeutically effective
amount of the polypeptide of the invention together with
a pharmaceutically acceptable diluent, carrier,
10 solubilizer, emulsifier, preservative and/or adjuvant.
The term "therapeutically effective amount" means an
amount which provides a therapeutic effect for a
specified condition and route of administration. The
composition may be in a liquid or lyophilized form and
15 comprises a diluent (Tris, acetate or phosphate buffers)
having various pH values and ionic strengths,
solubilizer such as Tween or Polysorbate, carriers such
as human serum albumin or gelatin, preservatives such as
thimerosal or benzyl alcohol, and antioxidants such as
20 ascorbic acid or sodium metabisulfite. Also encompassed
are compositions comprising OPG modified with water
soluble polymers to increase solubility or stability.
Compositions may also comprise incorporation of OPG into
liposomes, microemulsions, micelles or vesicles for
25 controlled delivery over an extended period of time.
Specifically, OPG compositions may comprise
incorporation into polymer matricies such as hydrogels,
silicones, polyethylenes, ethylene-vinyl acetate
copolymers, or biodegradable polymers. Examples of
30 hydrogels include polyhydroxyalkylmethacrylates (p-
HEMA), polyacrylamide, polymethacrylamide,
polyvinylpyrrolidone, polyvinyl alcohol and various
polyelectrolyte complexes. Examples of biodegradable
polymers include polylactic acid (PLA), polyglycolic

- 34 -

acid (PGA), copolymers of PLA and PGA, polyamides and copolymers of polyamides and polyesters. Other controlled release formulations include microcapsules, microspheres, macromolecular complexes and polymeric
5 beads which may be administered by injection.

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive
10 survey of component suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1980).

Compositions of the invention may be
15 administered by injection, either subcutaneous, intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled
20 in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic
25 acid compositions will be suitable for the delivery of part or all of the OPG coding region to cells and tissues as part of an anti-sense or gene therapy regimen.

30 Methods of Treatment

Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorous), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, osteocytes,

- 35 -

osteoblasts and osteoclasts, are involved in the dynamic
process by which bone is continually formed and
resorbed. Osteoblasts promote formation of bone tissue
whereas osteoclasts are associated with resorption.

5   Resorption, or the dissolution of bone matrix and
mineral, is a fast and efficient process compared to
bone formation and can release large amounts of mineral
from bone. Osteoclasts are involved in the regulation
of the normal remodeling of skeletal tissue and in

10   resorption induced by hormones. For instance,
resorption is stimulated by the secretion of parathyroid
hormone in response to decreasing concentrations of
calcium ion in extracellular fluids. In contrast,
inhibition of resorption is the principal function of

15   calcitonin. In addition, metabolites of vitamin D alter
the responsiveness of bone to parathyroid hormone and
calcitonin.

        After skeletal maturity, the amount of bone in
the skeleton reflects the balance (or imbalance) of bone

20   formation and bone resorption. Peak bone mass occurs
after skeletal maturity prior to the fourth decade.
Between the fourth and fifth decades, the equilibrium
shifts and bone resorption dominates. The inevitable
decrease in bone mass with advancing years starts

25   earlier in females than males and is distinctly
accelerated after menopause in some females (principally
those of Caucasian and Asian descent).

        Osteopenia is a condition relating generally
to any decrease in bone mass to below normal levels.

30   Such a condition may arise from a decrease in the rate
of bone synthesis or an increase in the rate of bone
destruction or both. The most common form of osteopenia
is primary osteoporosis, also referred to as
postmenopausal and senile osteoporosis. This form of

- 36 -

osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in bone resorption with a normal rate of bone formation. About 25 to 30 percent of all white females in the

5   United States develop symptomatic osteoporosis. A direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric fracture in women 45 years and older. Elderly males develop symptomatic osteoporosis between the ages of 50

10 and 70, but the disease primarily affects females.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been identified which may contribute to the condition. They include alteration in hormone levels accompanying aging

15 and inadequate calcium consumption attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary supplements in an attempt to retard the process. To date, however, an effective treatment

20 for bone loss does not exist.

The invention provides for a method of treating a bone disorder using a therapeutically effective amount of OPG. The bone disorder may be any disorder characterized by a net bone loss (osteopenia or

25 osteolysis). In general, treatment with OPG is anticipated when it is necessary to suppress the rate of bone resorption. Thus treatment may be done to reduce the rate of bone resorption where the resorption rate is above normal or to reduce bone resorption to below

30 normal levels in order to compensate for below normal levels of bone formation.

Conditions which are treatable with OPG include the following:

- 37 -

Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathryoidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of
5 osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.

Paget's disease of bone (osteitis deformans) in adults and juveniles
10 Osteomyelitis, or an infectious lesion in bone, leading to bone loss.

Hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignacies (multiple myeloma, lymphoma and leukemia), idiopathic
15 hypercalcemia, and hypercalcemia associated with hyperthryoidism and renal function disorders.

Osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic hepatic
20 and renal diseases.

Osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus and other conditions.
25 Bone loss due to rheumatoid arthritis.

Periodontal bone loss.

Osteolytic metastasis

It is understood that OPG may be used alone or in conjunction with other factors for the treatment of
30 bone disorders. In one embodiment, osteoprotegerein is used in conjunction with a therapeutically effective amount of a factor which stimulates bone formation. Such factors include but are not limited to the bone morphogenic factors designated BMP-1 through BMP-12,

- 38 -

transforming growth factor-β (TGF-β) and TGF-β family
members, interleukin-1 inhibitors, TNFα inhibitors,
parathyroid hormone and analogs thereof, parathyroid
related protein and analogs thereof, E series
5   prostaglandins, bisphosphonates (such as alendronate and
others), and bone-enhancing minerals such as fluoride
and calcium.

        The following examples are offered to more
10 fully illustrate the invention, but are not construed as
limiting the scope thereof.

15   EXAMPLE 1
              Identification and isolation of the
                      rat OPG cDNA

        Materials and methods for cDNA cloning and
20 analysis are described in Maniatis et al, _ibid_.
Polymerase chain reactions (PCR) were performed using a
Perkin-Elmer 9600 thermocycler using PCR reaction
mixture (Boehringer-Mannheim) and primer concentrations
specified by the manufacturer. In general, 25-50 μl
25 reactions were denatured at 94°C, followed by 20-40
cycles of 94°C for 5 seconds, 50-60°C for 5 seconds, and
72°C for 3-5 minutes. Reactions were the treated for 72
°C for 3-5 minutes. Reactions were then analyzed by gel
electrophoresis as described in Maniatis et al., _ibid_.
30   A cDNA library was constructed using mRNA
isolated from embryonic d20 intestine for EST analysis
(Adams et al. Science 252, 1651-1656 (1991)). Rat
embryos were dissected, and the entire developing small
and large intestine removed and washed in PBS. Total

- 39 -

cell RNA was purified by acid guanidinium thiocyanate-
phenol-chloroform extraction (Chomczynski and Sacchi
Anal. Biochem. 162, 156-159, (1987)). The poly (A+)
mRNA fraction was obtained from the total RNA
5   preparation by adsorption to, and elution from,
Dynabeads Oligo (dT)25 (Dynal Corp) using the
manufacturer's recommended procedures. A random primed
cDNA library was prepared using the Superscript Plasmid
System (Gibco BRL, Gaithersburg, Md). The random cDNA
10   primer containing an internal Not I restriction site was
used to initiate first strand synthesis and had the
following sequence:

5'-AAAGGAAGGAAAAAAGCGGCCGCTACANNNNNNNNNT-3' (SEQ ID NO:1)
                        Not I
15   For the first strand synthesis three separate
reactions were assembled that contained 2.5 µg of
poly(A) RNA and 120 ng, 360 ng or 1,080 ng of random
primer. After second strand synthesis, the reaction
products were separately extracted with a mixture of
20   phenol:choroform:isoamyl alcohol (25:24:1 ratio), and
then ethanol precipitated. The double strand (ds) cDNA
products of the three reactions were combined and
ligated to the following ds oligonucleotide adapter:

25   5'-TCGACCCACGCGTCCG-3' (SEQ ID NO:2)
      3'-GGGTGCGCAGGCp-5' (SEQ ID NO:3)

              After ligation the cDNA was digested to
completion with Not I, extracted with
30   phenol:chloroform:isoamyl (25:24:1) alcohol and ethanol
precipitated. The resuspended cDNA was then size
fractionated by gel filtration using premade columns
provided with the Superscript Plasmid System (Gibco BRL,
Gaithersburg, Md) as recommended by the manufacturer.

- 40 -

The two fractions containing the largest cDNA products
were pooled, ethanol precipitated and then directionally
ligated into Not I and Sal I digested pMOB vector DNA
(Strathmann et al, 1991). The ligated cDNA was
5   introduced into competent ElectroMAX DH10B E. coli
(Gibco BRL, Gaithersburg, MD) by electroporation. For
automated sequence analysis approximately 10,000
transformants were plated on 20cm x 20cm agar plates
containing ampicillin supplemented LB nutrient media.
10   The colonies that arose were picked and arrayed onto 96
well microtiter plates containing 200 ml of L-broth,
7.5% glycerol, and 50 µg/ml ampicillin. The cultures
were grown overnight at 37°C, a duplicate set of
microtiter plates were made using a sterile 96 pin
15   replicating tool, then both sets were stored at -80°C
for further analysis. For full-length cDNA cloning
approximately one million transformants were plated on
96 bacterial ampicillin plates containing about 10,000
clones each. The plasmid DNA from each pool was
20   separately isolated using the Qiagen Plasmid Maxi Kit
(Qiagen Corp.,Germany) and arrayed into 96 microtiter
plates for PCR analyses.

To sequence random fetal rat intestine cDNA
clones, glycerol stocks were thawed, and small aliquots
25   diluted 1:25 in distilled. Approximately 3.0 ul of
diluted bacterial cultures were added to PCR reaction
mixture (Boehringer-Mannheim) containing the following
oligonucleotides:

30   5'-TGTAAAACGACGGCCAGT-3' (SEQ ID NO:4)
          5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO:5)

The reactions were incubated in a thermocycler
(Perkin-Elmer 9600) with the following cycle conditions:

- 41 -

94 C for 2 minutes; 30 cycles of 94°C for 5 seconds,
50°C for 5 seconds, and 72°C for 3 minutes.; 72°C for 4
minutes. After incubation in the thermocycler, the
reactions were diluted with 2.0 mL of water. The

5 amplified DNA fragments were further purified using
Centricon columns (Princeton Separations) using the
manufacturer's recommended procedures. The PCR reaction
products were sequenced on an Applied Biosystems 373A
automated DNA sequencer using T3 primer (oligonucleotide

10 353-23; 5'-CAATTAACCCTCACTAAAGG-3') (SEQ ID NO:6) Taq
dye-terminator reactions (Applied Biosystems) following
the manufacturer's recommended procedures.

The resulting 5' nucleotide sequence obtained
from randomly picked cDNA clones translated and then

15 compared to the existing database of known protein
sequences using a modified version of the FASTA program
(Pearson et al. Meth. Enzymol. 183, (1990)). Translated
sequences were also analysed for the presence of a
specific cysteine-rich protein motif found in all known

20 members of the tumor necrosis factor receptor (TNFR)
superfamily (Smith et al. Cell 76, 959-962 (1994)),
using the sequence profile method of Gribskov et al.
(Proc. Natl. Acad. Sci. USA 83, 4355-4359 (1987)), as
modified by Luethy et al. (Protein Science 3, 139-146

25 (1994)).

Using the FASTA and Profile search data, an
EST, FRI-1 (Fetal Rat Intestine-1), was identified as a
possible new member of the TNFR superfamily. FRI-1
contained an approximately 600 bp insert with a LORF of

30 about 150 amino acids. The closest match in the
database was the human type II TNFR (TNFR-2). The
region compared showed an ~43% homology between TNFR-2
and FRI-1 over this 150 aa LORF. Profile analysis using
the first and second cysteine-rich repeats of the TNFR

- 42 -

superfamily yielded a Z score of ~8, indicating that the
FRI-1 gene possibly encodes a new family member.
To deduce the structure of the FRI-1 product, the fetal
rat intestine cDNA library was screened for full length
5   clones. The following oligonucleotides were derived
from the original FRI-1 sequence:

        5'-GCATTATGACCCAGAAACCGGAC-3' (SEQ ID NO:7)
        5'-AGGTAGCGCCCTTCCTCACATTC-3' (SEQ ID NO:8)
10

            These primers were used in PCR reactions to
screen 96 pools of plasmid DNA, each pool containing
plasmid DNA from 10,000 independent cDNA clones.
Approximately 1 ug of plasmid pool DNA was amplified in
15 a PCR reaction mixture (Boehringer-Mannheim) using a
Perkin-Elmer 96 well thermal cycler with the following
cycle conditions: 2 min at 94°C, 1 cycle; 15 sec at
94°C, then 45 sec at 65°C, 30 cycles; 7 min at 65°C, 1
cycle. PCR reaction products were analysed by gel
20 electrophoresis. 13 out of 96 plasmid DNA pools gave
rise to amplified DNA products with the expected
relative molecular mass.

            DNA from one positive pool was used to
transform competent ElectroMAX DH10B E. coli (Gibco BRL,
25 Gaithersburg, MD) as described above. Approximately
40,000 transformants were plated onto sterile
nitrocellulose filters (BA-85, Schleicher and Schuell),
and then screened by colony hybridization using a 32p-
dCTP labelled version of the PCR product obtained above.
30 Filters were prehybridized in 5X SSC, 50% deionized
formamide, 5X Denhardt's solution, 0.5% SDS, and 100
ug/ml denatured salmon sperm DNA for 2-4 hours at 42°C.
Filters were then hybridized in 5X SSC, 50% deionized
formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml

- 43 -

denatured salmon sperm DNA, and ~5 ng/ml of labelled
probe for ~18 hours at 42°C. The filters were then
washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at
55°C, and finally in 0.5X SSC for 10-15 min at 55°C.

5   Hybridizing clones were detected following
autoradiography, and then replated onto nitrocellulose
filters for secondary screening. Upon secondary
screening, a plasmid clone (pB1.1) was isolated, then
amplified in L-broth media containing 100 ug/ml

10   ampicillin and the plasmid DNA obtained. Both strands
of the 2.4 kb pB1.1 insert were sequenced.

The pB1.1 insert sequence was used for a FASTA
search of the public database to detect any existing
sequence matches and/or similarities. No matches to any

15   known genes or EST's were found, although there was an
approximate 45% similarity to the human and mouse TNFR-2
genes. A methionine start codon is found at bp 124 of
the nucleotide sequence, followed by a LORF encoding 401
aa residues that terminates at bp 1327. The 401 aa

20   residue product is predicted to have a hydrophobic
signal peptide of approximately 31 residues at its
N-terminus, and 4 potential sites of N-linked
glycosylation. No hydrophobic transmembrane spanning
sequence was identified using the PepPlot program

25   (Wisconsin GCG package, version 8.1). The deduced 401
aa sequence was then used to search the protein
database. Again, there were no existing matches,
although there appeared to be a strong similarity to
many members of the TNFR superfamily, most notably the

30   human and mouse TNFR-2. A sequence alignment of this
novel protein with known members of the TNFR-superfamily
was prepared using the Pileup program, and then modified
by PrettyPlot (Wisconsin GCG package, version 8.1).
This alignment shows a clear homology between the full

- 44 -

length FRI-1 gene product and all other TNFR family
members. The homologus region maps to the extracellular
domain of TNFR family members, and corresponds to the
three or four cysteine-rich repeats found in the ligand
5 binding domain of these proteins. This suggested that
the FRI-1 gene encoded a novel TNFR family member.
Since no transmembrane spanning region was detected we
predicted that this may be a secreted receptor, similar
to TNFR-1 derived soluble receptors (Kohno et al. Proc.
10 Natl. Acad. Sci. USA 87, 8331-8335 (1990)). Due to the
apparent biological activity of the FRI-1 gene (*vide
infra*), the product was named Osteoprotegerin (OPG).

15   EXAMPLE 2
            OPG mRNA Expression Patterns in Tissues

            Multiple human tissue northern blots
(Clonetech) were probed with a ^{32}P-dCTP labelled FRI-1
20 PCR product to detect the size of the human transcript
and to determine patterns of expression. Northern blots
were prehybridized in 5X SSPE, 50% formamide, 5X
Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured
salmon sperm DNA for 2-4 hr at 42°C. The blots were
25 then hybridized in 5X SSPE, 50% formamide, 2X Denhardt's
solution, 0.1% SDS, 100 μg/ml denatured salmon sperm
DNA, and 5 ng/ml labelled probe for 18-24 hr at 42°C.
The blots were then washed in 2X SSC for 10 min at RT,
1X SSC for 10 min at 50°C, then in 0.5X SSC for 10-15
30 min.

            Using a probe derived from the rat gene, a
predominant mRNA species with a relative molecular mass
of about 2.4 kb is detected in several tissues,
including kidney, liver, placenta, and heart. Highest

- 45 -

levels are detected in the kidney. A large mRNA species
of Mr 4.5 and 7.5 kb was detected in skeletal muscle and
pancreas. In human fetal tissue, kidney was found to
express relatively high levels of the 2.4 kb mRNA.
5   Using a human probe (vide infra), only the 2.4 kb
transcript is detected in these same tissues. In
addition, relatively high levels of the 2.4 kb
transcript was detected in the lymph node, thymus,
spleen and appendix. The size of the transcript
10 detected by both the rat and human Osteosprotegerin gene
is almost identical to the length of the rat pB1.1 FRI-1
insert, suggesting it was a full length cDNA clone.

EXAMPLE 3
15   Systemic delivery of OPG in transgenic mice

        The rat OPG clone pB1.1 was used as template
to PCR amplify the coding region for subcloning into an
ApoE-liver specific expression vector (Simonet et al. J.
20 Clin. Invest. 94, 1310-1319 (1994), and PCT Application
No. US94/11675 and co-owned U.S. Serial No. 08/221,767.
The following 5' and 3' oligonucleotide primers were
used for PCR amplification, respectively:

25 5'-GACTAGTCCCACAATGAACAAGTGGCTGTG-3' (SEQ ID NO:9)
5'-ATAAGAATGCGGCCGCTAAACTATGAAACAGCCCAGTGACCATTC-3'
(SEQ ID NO:10)

        The PCR reaction mixture (Boehringer-Mannheim)
30 was treated as follows: 94°C for 1 minute, 1 cycle; 94°C
for 20 sec, 62°C for 30 sec, and 74 C for 1 minute, 25
cycles. Following amplification, the samples were
purified over Qiagen PCR columns and digested overnight
with SpeI and NotI restriction enzymes. The digested

products were extracted and precipitated and subcloned into the ApoE promoter expression vector. Prior to microinjecting the resulting clone, HE-OPG, it was sequenced to ensure it was mutation-free.

5   The HE-OPG plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified plasmid DNA was digested with XhoI and Ase I, and the 3.6 kb transgene insert was purified by gel electrophoresis. The purified fragment was diluted to a

10   stock injection solution of 1 µg/ml in 5 mM Tris, pH 7.4, 0.2 mM EDTA. Single-cell embryos from BDF1 x BDF1-bred mice were injected essentially as described (Brinster et al., Proc. Natl. Acad. Sci. USA 82, 4338 (1985)), except that injection needles were beveled and

15   siliconized before use. Embryos were cultured overnight in a CO_2 incubator and 15 to 20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

             Following term pregnancy, 49 offspring were

20   obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. The target region for amplification was a 369 bp region of the human Apo E intron which was included in the expression

25   vector. The oligos used for PCR amplification were:

5'- GCC TCT AGA AAG AGC TGG GAC-3' (SEQ ID NO:11)
5'- CGC CGT GTT CCA TTT ATG AGC-3' (SEQ ID NO:12)

30   The conditions for PCR were: 94°C for 2 minute, 1 cycle; 94°C for 1 min, 63°C for 20 sec, and 72°C for 30 sec, 30 cycles. Of the 49 original offspring, 9 were identified as PCR positive transgenic founders.

- 47 -

At 8-10 weeks of age, five transgenic founders
(2, 11, 16, 17, and 28) and five controls (1, 12, 15,
18, and 30) were sacrificed for necropsy and
pathological analysis.   Liver was isolated from the
remaining 4 founders by partial hepatectomy. For
partial hepatectomy, the mice were anesthetized and a
lobe of liver was surgically removed. Total cellular
RNA was isolated from livers of all transgenic founders,
and 5 negative control littermates as described
(McDonald et al. Meth. Enzymol. 152, 219 (1987)).
Northern blot analysis was performed on these samples to
assess the level of transgene expression. Approximately
10ug of total RNA from each animal liver was resolved by
electrophoresis denaturing gels (Ogden et al. Meth.
Enzymol 152, 61 (1987)), then transferred to HYBOND-N
nylon membrane (Amersham), and probed with ^{32}P
dCTP-labelled pB1.1 insert DNA. Hybridization was
performed overnight at 42°C in 50% Formamide, 5 x SSPE,
0.5% SDS, 5 x Denhardt's solution, 100 µg/ml denatured
salmon sperm DNA and 2-4 x 10^6 cpm of labeled probe/ml
of hybridization buffer. Following hybridization, blots
were washed twice in 2 x SSC, 0.1% SDS at room
temperature for 5 min each, and then twice in 0.1 x SSC,
0.1% SDS at 55°C for 5-10 min each. Expression of the
transgene in founder and control littermates was
determined following autoradiography.

The northern blot data indicate that 7 of the
transgenic founders express detectable levels of the
transgene mRNA (animal #'s 2,11,16,17,22,33,and 45).
The negative control mice and one of the founders (#28)
expressed no transgene-related mRNA. Since OPG is
predicted to be a secreted protein, overexpression of
transgene mRNA should be a proxy for the level of
systemically delivered gene product. Of the PCR and

- 48 -

northern blot positive mice, animal 2, 17 and 22 expressed the highest levels of transgene mRNA, and may show more extensive biological effects on host cells and tissues.

5

EXAMPLE 4

Biological activity of OPG

10   Five of the transgenic mice (animals 2,11,16,17 and 28) and 5 control littermates (animals 1,12,15,18, and 30) were sacrificed for necropsy and pathological analysis using the following procedures: Prior to euthanasia, all animals had their

15   identification numbers verified, then were weighed, anesthetized and blood drawn. The blood was saved as both serum and whole blood for a complete serum chemistry and hematology panel. Radiography was performed just after terminal anesthesia by lethal CO2

20   inhalation, and prior to the gross dissection. Following this, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected included the liver, spleen, pancreas, stomach, duodenum, ileum, colon, kidney, reproductive

25   organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, eosphagus, thyroid, jejunem, cecum, rectum, adrenals, urinary bladder, and skeletal muscle. Prior to fixation the whole organ weights were determined for the liver, stomach, kidney, adrenals,

30   spleen, and thymus. After fixation the tissues were processed into paraffin blocks, and 3 um sections were obtained. Bone tissue was decalcified using a formic acid solution, and all sections were stained with hematoxylin and eosin. In addition, staining with

- 49 -

Gomori's reticulin and Masson's trichrome were performed
on certain tissues. Enzyme histochemistry was performed
to determine the expression of tartrate resistant acid
phosphatase (TRAP), an enyzme highly expressed by
5   osteoclasts, multinucleated bone-resorbing cells of
monocyte-macrophage lineage. Immunohistochemistry for
BrdU and F480 monocyte-macrophage surface antigen was
also performed to detect replicating cells and cells of
the monocyte-macrophage lineage, respectively. To
10 detect F480 surface antigen expression, formalin fixed,
paraffin embedded 4μm sections were deparaffinized and
hydrated to deionized water. The sections were quenched
with 3% hydrogen peroxide, blocked with Protein Block
(Lipshaw, Pittsburgh, PA), and incubated in rat
15 monoclonal anti-mouse F480 (Harlan, Indianapolis, IN).
This antibody was detected by biotinylated rabbit anti-
rat immunoglobulins, peroxidase conjugated strepavidin
(BioGenex San Ramon, CA) with DAB as chromagen (BioTek,
Santa Barbara, CA). Sections were counterstained with
20 hematoxylin.

Upon gross dissection and observation of
visceral tissues, no abnormalities were found in the
transgene expressors or control littermates. Analysis
of organ weight indicate that spleen size increased by
25 approximately 38% in the transgenic mice relative to
controls. There was a slight enlargement of platelet
size and increased circulating unstained cells in the
transgene expressors. There was a marginal decrease in
platelet levels in the transgene expressors. In
30 addition, the serum uric acid, urea nitrogen, and
alkaline phosphatase levels all trended lower in the
transgene expressors. The expressors were found to have
increased radiodensity of the skeleton, including long
bones (femurs), vertebrae, and flat bones (pelvis). The

- 50 -

relative size of femurs in the expressors were not
different from the the control mice.

Histological analysis of stained sections of
bone from the OPG expressors show severe osteopetrosis
5   with the presence of cartilage remnants from the primary
spongiosa seen within bone trabeculae in the diaphysis
of the femur. A clearly defined cortex was not
identifiable in the sections of femur. In normal
animals, the central diaphysis is filled with bone
10   marrow. Sections of vertebra also show osteopetrotic
changes implying that the OPG-induced skeletal changes
were systemic. The residual bone marrow showed
predominantly myeloid elements. Megakaryocytes were
present. Reticulin stains showed no evidence for
15   reticulin deposition. Immunohistochemistry for F480, a
cell surface antigen expressed by cells of monocyte-
macrophage derivation in the mouse, showed the presence
of F480 positive cells in the marrow spaces. Focally,
flattened F480 positive cells could be seen directly
20   adjacent to trabecular bone surfaces.

The mesenchymal cells lining the bony
trabeculae were flattened and appeared inactive. Based
on H&E and TRAP stains, osteoclasts were rarely found on
the trabecular bone surfaces in the OPG expressors. In
25   contrast, osteoclasts and/or chondroclasts were seen in
the region of the growth plate resorbing cartilage, but
their numbers may be reduced compared to controls. Also,
osteoclasts were present on the cortical surface of the
metaphysis where modelling activity is usually robust.
30   The predominant difference between the expressors and
controls was the profound decrease in trabecular
osteoclasts, both in the vertebrae and femurs. The
extent of bone accumulation was directly correlated with

- 51 -

the level of OPG transgene mRNA detected by northern
blotting of total liver RNA.

　　　　The spleens from the OPG expressors had an
increased amount of red pulp with the expansion due to
5　　increased hematopoiesis. All hematopoietic lineages are
represented. F480 positive cells were present in both
control and OPG expressors in the red pulp. Two of the
expressors (2 and 17)had foci of extramedullary
hematopoiesis within the liver and this is likely due to
10　the osteopetrotic marrow.

　　　　There were no observable abnormalities in the
thymus, lymph nodes, gastrointestinal tract, pancreato-
hepatobiliary tract, respiratory tract, reproductive
system, genito-urinary system, skin, nervous system,
15　heart and aorta, breast, skeletal muscle and fat.

EXAMPLE 5

Isolation of mouse and human OPG cDNA

20

　　　　A cDNA clone corresponding to the 5' end of
the mouse OPG mRNA was isolated from a mouse kidney cDNA
library (Clontech) by PCR amplification. The
oligonucleotides were derived from the rat OPG cDNA
25　sequence and are shown below:

5'-ATCAAAGGCAGGGCATACTTCCTG-3' (SEQ ID NO:13)
5'-GTTGCACTCCTGTTTCACGGTCTG-3' (SEQ ID NO:14)

30　5'-CAAGACACCTTGAAGGGCCTGATG-3' (SEQ ID NO:15)
5'-TAACTTTTACAGAAGAGCATCAGC-3' (SEQ ID NO:16)

5'-AGCGCGGCCGCATGAACAAGTGGCTGTGCTGCG-3' (SEQ ID NO:17)
5'-AGCTCTAGAGAAACAGCCCAGTGACCATTCC-3' (SEQ ID NO:18)

- 52 -

The partial and full-length cDNA products obtained in this process were sequenced. The full-length product was digested with Not I and Xba I, then 5 directionally cloned into the plasmid vector pRcCMV (Invitrogen). The resulting plasmid was named pRcCMV-Mu-OPG. The nucleotide sequence of the cloned product was compared to the rat OPG cDNA sequence. Over the 1300 bp region spanning the OPG LORF, the rat and mouse 10 DNA sequences are approximately 88% identical. The mouse cDNA sequence contained a 401 aa LORF, which was compared to the rat OPG protein sequence and found to be ~94% identical without gaps. This indicates that the mouse cDNA sequence isolated encodes the murine OPG 15 protein, and that the sequence and structure has been highly conserved throughout evolution. The mouse OPG protein sequence contains an identical putative signal peptide at its N-terminus, and all 4 potential sites of N-linked glycosylation are conserved.

20 A partial human OPG cDNA was cloned from a human kidney cDNA library using the following rat-specific oligonucleotides:

5'-GTG AAG CTG TGC AAG AAC CTG ATG-3'
25 (SEQ ID NO:19)
5'-ATC AAA GGC AGG GCA TAC TTC CTG-3'
(SEQ ID NO:20)

This PCR product was sequenced and used to 30 design primers for amplifying the 3' end of the human cDNA using a human OPG genomic clone in lambda as template:

5'-TCCGTAAGAAACAGCCCAGTGACC-3' (SEQ ID NO:29)

- 53 -

5'-CAGATCCTGAAGCTGCTCAGTTTG-3' (SEQ ID NO:21)

The amplified PCR product was sequenced, and
together with the 5' end sequence, was used to design 5'
5   and 3' human-specific primers useful for amplifying the
entire human OPG cDNA coding sequences:

5'-AGCGCGGCCGCGGGGACCACAATGAACAAGTTG-3' (SEQ ID NO:22)
5'-AGCTCTAGAATTGTGAGGAAACAGCTCAATGGC-3' (SEQ ID NO:23)
10

The full-length human PCR product was
sequenced, then directionally cloned into the plasmid
vector pRcCMV (Invitrogen) using Not I and Xba I. The
resulting plasmid was named pRcCMV-human OPG. The
15   nucleotide sequence of the cloned product was compared
to the rat and mouse OPG cDNA sequences. Over the 1300
bp region spanning the OPG LORF, the rat and mouse DNA
sequences are approximately 78-88% identical to the
human OPG cDNA. The human OPG cDNA sequence also
20   contained a 401 aa LORF, and it was compared to the rat
and mouse protein sequences. The predicted human OPG
protein is approximatlely 85% identical, and ~90%
identical to the rat and mouse proteins, respectively.
Sequence alignment of rat, mouse and human proteins show
25   that they have been highly conserved during evolution.
The human protein is predicted to have a N-terminal
signal peptide, and 5 potential sites of N-linked
glycosylation, 4 of which are conserved between the rat
and mouse OPG proteins.
30   The DNA and predicted amino acid sequence of
mouse OPG is shown in Figure 9A and 9B (SEQ ID NO:122).
The DNA and predicted amino acid sequence of human OPG
is shown in Figure 9C an 9D (SEQ ID NO:124). A

- 54 -

comparison of the rat, mouse and human OPG amino acid
sequences is shown in Figure 9E and 9F.

    Isolation of additional human OPG cDNA clones
revealed the presence of a G to C base change at
5   position 103 of the DNA sequence shown in Figure 9C.
This nucleotide change results in substitution of an
asparagine for a lysine at position 3 of the amino acid
sequence shown in Figure 9C. The remainder of the
sequence in clones having this change was identical to
10 that in Figure 9C and 9D.

                    EXAMPLE 6
        OPG three-dimensional structure modelling
15

    The amino-terminal portion of OPG has homology
to the extracellular portion of all known members of the
TNFR superfamily (Figure 1C). The most notable motif in
this region of TNFR-related genes is an ~40 amino acid,
20 cysteine-rich repeat sequence which folds into distinct
structures (Banner et al. Cell 73, 431-445 (1993)).
This motif is usually displayed in four (range 3-6)
tandem repeats (see Figure 1C), and is known to be
involved in ligand binding (Beutler and van Huffel
25 Science 264, 667-663 (1994)). Each repeat usually
contains six interspaced cysteine residues, which are
involved in forming three intradomain disulfide bonds,
termed SS1, SS2, and SS3 (Banner et al., ibid). In some
receptors, such as TNFR2, CD30 and CD40, some of the
30 repeat domains contain only two intrachain disulfide
bonds (SS1 and SS3).

    The human OPG protein sequence was aligned to
a TNFR1 extracellular domain profile using methods
described by Luethy, et al., ibid, and the results were

- 55 -

graphically displayed using the PrettyPlot program from
the Wisconsin Package, version 8.1 (Genetics Computer
Group, Madison, WI) (Figure 10). The alignment
indicates a clear conservation of cysteine residues
5　　involved in formation of domains 1-4. This alignment
was then used to construct a three-dimensional (3-D)
model of the human OPG N-terminal domain using the known
3-D structure of the extracellular domain of p55 TNFR1
(Banner et al., ibid) as the template. To do this the
10　　atomic coordinates of the peptide backbone and side
chains of identical residues were copied from the
crystal structure coordinates of TNFR1. Following this,
the remaining coordinates for the insertions and
different side chains were generated using the LOOK
15　　program (Molecular Applications Group, Palo Alto, CA).
The 3-D model was then refined by minimizing its
conformational energy using LOOK.

　　　　　By analogy with other TNFR family members, it
is assumed that OPG binds to a ligand. For the purpose
20　　of modelling the interaction of OPG with its ligand, the
crystal structure of TNF-β was used to simulate a 3-D
representation of an "OPG ligand". This data was
graphically displayed (see Figure 11) using Molscript
(Kraulis, J. Appl. Cryst. 24, 946-950, 1991). A model
25　　for the OPG/ligand complex with 3 TNFβ and 3 OPG
molecules was constructed where the relative positions
of OPG are identical to TNFR1 in the crystal structure.
This model was then used to find the residues of OPG
that could interact with its ligand using the following
30　　approach: The solvent accessible area of all residues
in the complex and one single OPG model were calculated.
The residues that have different accessibility in the
complex than in the monomer are likely to interact with
the ligand.

- 56 -

The human and mouse OPG amino acid sequences were realigned using this information to highlight sequences comprising each of the cysteine rich domains 1-4 (Figure 12A and 12B). Each domain has individual structural characteristics which can be predicted:

Domain 1

Contains 4 cysteines involved in SS2 (C41 to C54) and SS3 (C44 to C62) disulfide bonds. Although no SS1 bond is evident based on disulfide bridges, the conserved tyrosine at position 28 is homologous to Y20 in TNFR1, which is known to be involved in interacting with H66 to aid in domain formation. OPG has a homologous histidine at position 75, suggesting OPG Y28 and H75 stack together in the native protein, as do the homologous residues in TNFR1. Therefore, both of these residues may indeed be important for biological activity, and N-terminal OPG truncations up to and beyond Y28 may have altered activity. In addition, residues E34 and K43 are predicted to interact with a bound ligand based on our 3-dimensional model.

Domain 2

Contains six cysteines and is predicted to contain SS1 (C65 to C80), SS2 (C83 to C98) and SS3 (C87 to C105) disulfide bonds. This region of OPG also contains an region stretching from P66-Q91 which aligns to the portion of TNFR1 domain 2 which forms close contacts with TNFβ (see above), and may interact with an OPG ligand. In particular residues P66, H68, Y69, Y70, T71, D72, S73, H75, T76, S77, D78, E79, L81, Y82, P85, V86, K88, E89, L90, and Q91 are predicted to interact with a bound ligand based on our structural data.

- 57 -

Domain 3

Contains 4 cysteines involved in SS1 (C107 to
C 118) and SS3 (C124 to C142) disulfide bonds, but not
an SS2 bond. Based on our structural data, residues
5   E115, L118 and K119 are predicted in to interact with an
OPG ligand.

Domain 4

Contains 4 cysteines involved in SS1 (C145 to
10   C160) and SS3 (C166 to C185) disulfide bonds, but not an
SS2 bond, similar to domain 3. Our structural data
predict that E153 and S155 interact with an OPG ligand.

Thus, the predicted structural model for OPG
15   identifies a number of highly conserved residues which
are likely to be important for its biological activity.

EXAMPLE 7
20   Production of recombinant secreted
OPG protein in mammalian cells

To determine if OPG is actually a secreted
protein, mouse OPG cDNA was fused to the human IgG1 Fc
25   domain as a tag (Capon et al. Nature 337, 525-531
(1989)), and expressed in human 293 fibroblasts. Fc
fusions were carried out using the vector pFc-A3. pFc-
A3 contains the region encoding the Fc portion of human
immunoglobulin IgG-γ1 heavy chain (Ellison et al. ibid)
30   from the first amino acid of the hinge domain (Glu-99)
to the carboxyl terminus and is flanked by a 5'-NotI
fusion site and 3'-SalI and XbaI sites. The plasmid was
constructed by PCR amplification of the human spleen
cDNA library (Clontech). PCR reactions were in a final

- 58 -

volume of 100 µl and employed 2 units of Vent DNA
polymerase (New England Biolabs) in 20 mM Tris-HCl (pH
8.8), 10 mM KCl, 10 µM (NH$_4$)2SO$_4$, 2 mM MgSO$_4$, 0.1% Triton
X-100 with 400 µM each dNTP and 1 ng of the cDNA library

5   to be amplified together with 1 µM of each primer.
Reactions were initiated by denaturation at 95°C for 2
min, followed by 30 cycles of 95°C for 30 s, 55°C for 30
s, and 73°C for 2 min. The 5' primer

5' ATAGCGGCCGCTGAGCCCAAATCTTGTGACAAAACTCAC 3' (SEQ

10   ID NO:24)

incorporated a NotI site immediately 5' to the first
residue (Glu-99) of the hinge domain of IgG-γ1. The 3'
primer

5'-TCTAGAGTCGACTTATCATTTACCCGGAGACAGGGAGAGGCTCTT-3'

15   (SEQ ID NO:25)

incorporated SalI and XbaI sites. The 717-bp PCR
product was digested with NotI and SalI, isolated by
electrophoresis through 1% agarose (FMC Corp.),purified
by the Geneclean procedure (BIO 101, Inc.) and cloned

20   into NotI, SalI-digested pBluescript II KS vector
(Stratagene). The insert in the resulting plasmid, pFc-
A3, was sequenced to confirm the fidelity of the PCR
reaction.

The cloned mouse cDNA in plasmid pRcCMV-MuOPG

25   was amplified using the following two sets of primer
pairs:

Pair 1

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID

30   NO:26)

5'-CCTCTGCGGCCGCTAAGCAGCTTATTTTCACGGATTGAACCTG-3' (SEQ
ID NO:27)

- 59 -

Pair 2

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID NO:28)

5'-CCTCTGCGGCCGCTGTTGCATTTCCTTTCTG-3' (SEQ ID NO:30)

5

The first pair amplifies the entire OPG LORF, and creates a NotI restriction site which is compatible with the in-frame Not I site in Fc fusion vector pFcA3. pFcA3 was prepared by engineering a NotI restriction
10 site 5' to aspartic acid reside 216 of the human IgG1 Fc cDNA. This construct introduces a linker which encodes two irrelevant amino acids which span the junction between the OPG protein and the IgG Fc region. This product, when linked to the Fc portion, would encode all
15 401 OPG residues directly followed by all 227 amino acid residues of the human IgG1 Fc region (Fl.Fc). The second primer pair amplifies the DNA sequences encoding the first 180 amino acid residues of OPG, which encompasses its putative ligand binding domain. As
20 above, the 3' primer creates an artificial Not I restriction site which fuses the C-terminal truncated OPG LORF at position threonine 180 directly to the IgG1 Fc domain (CT.fc).

The amino acid sequence junction linking OPG
25 residue 401 and aseptic acid residue 221 of the human Fc region can be modified as follows: The DNA encoding residues 216-220 of the human Fc region can be deleted as described below, or the cysteine residue corresponding to C220 of the human Fc region can be
30 mutated to either serine or alanine. OPF-Fc fusion protein encoded by these modifed vectors can be transfected into human 293 cells, or CHO cells, and recombinant OPG-Fc fusion protein purified as described below.

- 60 -

Both products were directionally cloned into the plasmid vector pCEP4 (Invitrogen). pCEP4 contains the Epstein-Barr virus origin of replication, and is capable of episomal replication in 293-EBNA-1 cells.

5   The parent pCEP4, and pCEP4-Fl.Fc and pCEP4-CT.Fc vectors were lipofected into 293-EBNA-1 cells using the manufacturer's recommended methods. The transfected cells were then selected in 100 μg/ml hygromycin to select for vector expression, and the resulting drug-

10 resistant mass cultures were grown to confluence. The cells were then cultured in serum-free media for 72 hr, and the conditioned media removed and analysed by SDS-PAGE. A silver staining of the polyacrylamide gel detects the major conditioned media proteins produced by

15 the drug resistant 293 cultures. In the pCEP4-Fl.Fc and the pCEP4-CT.Fc conditioned media, unique bands of the predicted sizes were abundantly secreted (see Figures 13B and 13C). The full-length Fc fusion protein accumulated to a high concentration, indicating that it

20 may be stable. Both Fc fusion proteins were detected by anti-human IgG1 Fc antibodies (Pierce) on western blots, indicating that they are recombinant OPG products.

The full length OPG-Fc fusion protein was purified by Protein-A column chromatography (Pierce)

25 using the manufacturers recommended procedures. The protein was then subjected to N-terminal sequence analysis by automated Edman degradation as essentially described by Matsudaira et al. (J. Biol. Chem. 262, 10-35 (1987)). The following amino acid sequence was read

30 after 19 cycles:

NH2-E T L P P K Y L H Y D P E T G H Q L L-CO2H
(SEQ ID NO:31)

- 61 -

This sequence was identical to the predicted mouse OPG amino acid sequence beginning at amino acid residue 22, suggesting that the natural mammalian leader cleavage site is between amino acid residues Q21-E22,

5   not between Y31-D32 as originally predicted. The expression experiments performed in 293-EBNA cells with pCEP4-Fl.Fc and pCEP4-CT.Fc demonstrate that OPG is a secreted protein, and may act systemically to bind its ligand.

10   Procedures similar to those used to construct and express the muOPG[22-180]-Fc and muOPG[22-401]-Fc fusions were employed for additional mouse and human OPG-Fc fusion proteins.

Murine OPG cDNA encoding amino acids 1-185

15   fused to the Fc region of human IgG1 [muOPG Ct(185).Fc] was constructed as follows. Murine OPG cDNA from plasmid pRcCMV Mu Osteoprotegerin (described in Example 5) was amplified using the following primer pair in a polymerase chain reaction as described above:

20

1333-82:

      5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:32)
1333-80:

      5'-CCT CTG CGG CCG CAC ACA CGT TGT CAT GTG TTG C-3'

25   (SEQ ID NO:33)

This primer pair amplifies the murine OPG cDNA region encoding amino acid residues 63-185 (corresponding to bp 278-645) of the OPG reading frame

30   as shown in Figure 9A. The 3' primer contains a Not I restriction site which is compatible with the in-frame Not I site of the Fc fusion vector pFcA3. The product also spans a unique EcoRI restriction site located at bp 436. The amplified PCR product was purified, cleaved

- 62 -

with NotI and EcoRI, and the resulting EcoRI-NotI
restriction fragment was purified. The vector pCEP4
having the murine 1-401 OPG-Fc fusion insert was cleaved
with EcoRI and NotI, purified, and ligated to the PCR
5   product generated above. The resulting pCEP4-based
expression vector encodes OPG residues 1-185 directly
followed by all 227 amino acid residues of the human
IgG1 Fc region. The murine OPG 1-185.Fc fusion vector
was transfected into 293 cells, drug selected, and
10   conditioned media was produced as described above. The
resulting secreted murine OPG 1-185.Fc fusion product
was purified by Protein-A column chromatography (Pierce)
using the manufacturers recommended procedures.

15   Murine OPG DNA encoding amino acid residues 1-
194 fused to the Fc region of human IgG1 (muOPG
Ct(194).Fc) was constructed as follows. Mouse OPG cDNA
from plasmid pRcCMV Mu-Osteoprotegerin was amplified
using the following primer pairs:
20

1333-82:
     5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:34)
1333-81:
     5'-CCT CTG CGG CCG CCT TTT GCG TGG CTT CTC TGT T-3'
25   (SEQ ID NO:35)

             This primer pair amplifies the murine OPG cDNA
region encoding amino acid residues 70-194
(corresponding to bp 298-672) of the OPG reading frame.
30   The 3' primer contains a Not I restriction site which is
compatible with the in-frame Not I site of the Fc fusion
vector pFcA3. The product also spans a unique EcoRI
restriction site located at bp 436. The amplified PCR
product was cloned into the murine OPG[1-401] Fc fusion

- 63 -

vector as described above. The resulting pCEP4-based
expression vector encodes OPG residues 1-194 directly
followed by all 227 amino acid residues of the human
IgG1 Fc region. The murine OPG 1-194.Fc fusion vector

5   was transfected into 293 cells, drug selected, and
conditioned media was produced. The resulting secreted
fusion product was purified by Protein-A column
chromatography (Pierce) using the manufacturers
recommended procedures.

10

            Human OPG DNA encoding amino acids 1-401 fused
to the Fc region of human IgG1 was constructed as
follows. Human OPG DNA in plasmid pRcCMV-hu
osteoprotegerin (described in Example 5) was amplified

15   using the following oligonucleotide primers:

1254-90:
        5'CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'
(SEQ ID NO:36)

20

1254-95:
        5'-CCT CTG CGG CCG CTA AGC AGC TTA TTT TTA CTG AAT GG-3'
(SEQ ID NO:37)

25   The resulting PCR product encodes the full-
length human OPG protein and creates a Not I restriction
site which is compatible with the in-frame Not I site Fc
fusion vector FcA3. The PCR product was directionally
cloned into the plasmid vector pCEP4 as described above.

30   The resulting expression vector encodes human OPG
residues 1-401 directly followed by 227 amino acid
residues of the human IgG1 Fc region. Conditioned media
from transfected and drug selected cells was produced
and the huOPG Fl.Fc fusion product was purified by

- 64 -

Protein-A column chromatography (Pierce) using the
manufacturers recommended procedures.

Human OPG DNA encoding amino acid residues 1-
5   201 fused to the Fc region of human IgG1 [huOPG
Ct(201).Fc] was constructed as follows. The cloned human
OPG cDNA from plasmid pRrCMV-hu osteoprotegerin was
amplified by PCR using the following oligonucleotide
primer pair:

10

1254-90:

    5'-CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT
G-3' (SEQ ID NO:38)
1254-92:

15   5'-CCT CTG CGG CCG CCA GGG TAA CAT CTA TTC CAC-3'
(SEQ ID NO:39)

This primer pair amplifies the human OPG cDNA
region encoding amino acid residues 1-201 of the OPG
20   reading frame, and creates a Not I restriction site at
the 3' end which is compatable with the in-frame Not I
site Fc fusion vector FcA3. This product, when linked
to the Fc portion, encodes OPG residues 1-201 directly
followed by all 221 amino acid residues of the human
25   IgG1 Fc region. The PCR product was directionally
cloned into the plasmid vector pCEP4 as described above.
Conditioned media from transfected and drug selected
cells was produced, and the hu OPG Ct(201).Fc fusion
products purified by Protein-A column chromatography
30   (Pierce) using the manufacturer's recommended
procedures.

The following procedures were used to
construct and express unfused mouse and human OPG.

- 65 -

A plasmid for mammalian expression of full-length murine OPG (residues 1-401) was generated by PCR amplification of the murine OPG cDNA insert from pRcCMV Mu-Osteoprotegerin and subcloned into the expression

5   vector pDSRα (DeClerck et. atl. J. Biol. Chem. 266, 3893 (1991)). The following oligonucleotide primers were used:

1295-26:

10   5'-CCG AAG CTT CCA CCA TGA ACA AGT GGC TGT GCT GC-3' (SEQ ID NO:40)

1295-27:

         5'-CCT CTG TCG ACT ATT ATA AGC AGC TTA TTT TCA CGG
15   ATT G-3' (SEQ ID NO:41)

The murine OPG full length reading frame was amplified by PCR as described above. The PCR product was purified and digested with restriction endonucleases

20   Hind III and Xba I (Boehringer Mannheim, Indianapolis, IN) under the manufacturers recommended conditions, then ligated to Hind III and Xba I digested pDSRα. Recombinant clones were detected by restriction endonuclease digestion, then sequenced to ensure no

25   mutations were produced during the PCR amplification steps.

The resulting plasmid, pDSRα-muOPG was introduced into Chinese hamster ovary (CHO) cells by calcium mediated transfection (Wigler et al. Cell 11,

30   233 (1977)). Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the murine OPG recombinant protein was monitored by western blot analysis of CHO

- 66 -

cell conditioned media. High expressing cells were selected, and OPG expression was further amplified by treatment with methotrexate as described (DeClerck et al., idid). Conditioned media from CHO cell lines was

5 produced for further purification of recombinant secreted murine OPG protein.

A plasmid for mammalian expression of full-length human OPG (amino acids 1-401) was generated by

10 subcloning the cDNA insert in pRcCMV-hu Osteoprotegerin directly into vector pDSRα (DeClerck et al., ibid). The pRcCMV-OPG plasmid was digested to completion with Not I, blunt ended with Klenow, then digested to completion with Xba I. Vector DNA was digested with Hind III,

15 blunt ended with Klenow, then digested with Xba I, then ligated to the OPG insert. Recombinant plasmids were then sequenced to confirm proper orientation of the human OPG cDNA.

The resulting plasmid pDSRα-huOPG was

20 introduced into Chinese hamster ovary (CHO) cells as described above. Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones were isolated. Expression of the human OPG recombinant

25 protein was monitored by western blot analysis of CHO cell conditioned media. High expressing clones were selected, and OPG expression was further amplified by treatment with methotrexate. Conditioned media from CHO cell lines expressing human OPG was produced for protein

30 purification.

Expression vectors for murine OPG encoding residues 1-185 were constructed as follows. Murine OPG

- 67 -

cDNA from pRcCMV-Mu OPG was amplified using the
following oligonucleotide primers:

1333-82:

5   5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:42)
1356-12:

5'-CCT CTG TCG ACT TAA CAC ACG TTG TCA TGT GTT
GC-3' (SEQ ID NO:43)

10   This primer pair amplifies the murine OPG cDNA
region encoding amino acids 63-185 of the OPG reading
frame (bp 278-645) and contains an artificial stop codon
directly after the cysteine codon (C185), which is
followed by an artificial Sal I restriction endonuclease
15   site. The predicted product contains an internal Eco RI
restriction site useful for subcloning into a pre-
existing vector. After PCR amplification, the resulting
purified product was cleaved with Eco RI and Sal I
restriction endonucleases, and the large fragment was
20   gel purified. The purified product was then subcloned
into the large restriction fragment of an Eco RI and Sal
I digest of pBluescript-muOPG Fl.Fc described above.
The resulting plasmid was digested with Hind III and Xho
I and the small fragment was gel purified. This
25   fragment, which contains a open reading frame encoding
residues 1-185 was then subcloned into a Hind III and
Xho I digest of the expression vector pCEP4. The
resulting vector, pmuOPG [1-185], encodes a truncated
OPG polypeptide which terminates at a cysteine residue
30   located at position 185. Conditioned media from
transfected and drug selected cells was produced as
described above.

- 68 -

1333-82:

    5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:44)

1356-13:

    5'-CCT CTG TCG ACT TAC TTT TGC GTG GCT TCT CTG
TT-3' (SEQ ID NO:45)

       This primer pair amplifies the murine OPG cDNA
region encoding amino acids 70-194 of the OPG reading
frame (bp 298-672) and contains an artificial stop codon
directly after the lysine codon (K194), which is
followed by an artificial Sal I restriction endonuclease
site. The predicted product contains an internal Eco RI
restriction site useful for subcloning into a pre-
existing vector. After PCR amplification, the resulting
purified product was cleaved with Eco RI and Sal I
restriction endonucleases, and the large fragment was
gel purified. The purified product was then subcloned
into the large restriction fragment of an Eco RI and Sal
I digest of pBluescript-muOPG F1.Fc described above.
The resulting plasmid was digested with Hind III and Xho
I and the small fragment was gel purified. This
fragment, which contains a open reading frame encoding
residues 1-185 was then subcloned into a Hind III and
Xho I digest of the expression vector pCEP4. The
resulting vector, pmuOPG [1-185], encodes a truncated
OPG polypeptide which terminates at a lysine at position
194. Conditioned media from transfected and drug
selected cells was produced as described above.

       Several mutations were generated at the 5' end
of the huOPG [22-401]-Fc gene that introduce either
amino acid substitutions, or deletions, of OPG between
residues 22 through 32. All mutations were generated

with the "QuickChange™ Site-Directed Mutagenesis Kit"
(Stratagene, San Diego, CA) using the manfacturer's
recommended conditions. Briefly, reaction mix
containing huOPG [22-401]-Fc plasmid DNA template and
5   mutagenic primers were treated with Pfu polymerase in
the presence of deoxynucleotides, then amplified in a
thermocycler as described above. An aliqout of the
reaction is then transfected into competent E. coli XL1-
Blue by heatshock, then plated. Plasmid DNA from
10 transformants was then sequenced to verify mutations.
            The following primer pairs were used to delete
residues 22-26 of the human OPG gene, resulting in the
production of a huOPG [27-401]-Fc fusion protein:

15 1436-11:
            5'-TGG ACC ACC CAG AAG TAC CTT CAT TAT GAC-3'
    (SEQ ID NO:140)

    1436-12:
20   5'-GTC ATA ATG AAG GTA CTT CTG GGT GGT CCA-3'
    (SEQ ID NO:141)

            The following primer pairs were used to delete
residues 22-28 of the human OPG gene, resulting in the
25 production of a huOPG [29-401]-Fc fusion protein:

    1436-17:
            5'-GGA CCA CCC AGC TTC ATT ATG ACG AAG AAA C-3'
    (SEQ ID NO:142)

30
    1436-18:
            5'-GTT TCT TCG TCA TAA TGA AGC TGG GTG GTC C-3'
    (SEQ ID NO:143)

- 70 -

The following primer pairs were used to delete residues 22-31 of the human OPG gene, resulting in the production of a huOPG [32-401]-Fc fusion protein:

5

1436-27:

    5'-GTG GAC CAC CCA GGA CGA AGA AAC CTC TC-3'
(SEQ ID NO:144)

10   1436-28:

    5'-GAG AGG TTT CTT CGT CCT GGG TGG TCC AC-3'
(SEQ ID NO:145)

The following primer pairs were used to change
15 the codon for tyrosine residue 28 to phenylalanine of the human OPG gene, resulting in the production of a huOPG [22-401]-Fc Y28F fusion protein:

1436-29:

20   5'-CGT TTC CTC CAA AGT TCC TTC ATT ATG AC-3'
(SEQ ID NO:146)

1436-30:

    5'-GTC ATA ATG AAG GAA CTT TGG AGG AAA CG-3'
25 (SEQ ID NO:147)

The following primer pairs were used to change the codon for proline residue 26 to alanine of the human OPG gene, resulting in the production of a huOPG [22-
30 401]-Fc P26A fusion protein:

1429-83:

    5'-GGA AAC GTT TCC TGC AAA GTA CCT TCA TTA TG-3
(SEQ ID NO:148)

- 71 -

1429-84:

    5'-CAT AAT GAA GGT ACT TTG CAG GAA ACG TTT CC-3'
(SEQ ID NO:149)

5

    Each resulting muOPG [22-401]-Fc plasmid
containing the appropriate mutation was then transfected
into human 293 cells, the mutant OPG-Fc fusion protein
purified from conditioned media as described above. The
10 biological activity of each protein was assessed the in
vitro osteoclast forming assay described in Example 11.

EXAMPLE 8
15 Expression of OPG in E. coli

A. Bacterial Expression Vectors

pAMG21

20   The expression plasmid pAMG21 can be derived
from the Amgen expression vector pCFM1656 (ATCC #69576)
which in turn be derived from the Amgen expression
vector system described in US Patent No. 4,710,473. The
pCFM1656 plasmid can be derived from the described
25 pCFM836 plasmid (Patent No. 4,710,473) by: (a)
destroying the two endogenous NdeI restriction sites by
end filling with T4 polymerase enzyme followed by blunt
end ligation; (b) replacing the DNA sequence between the
unique AatII and ClaI restriction sites containing the
30 synthetic P$_L$ promoter with a similar fragment obtained
from pCFM636 (patent No. 4,710,473) containing the PL
promoter

- 72 -

```
     AatII
  5' 	CTAATTCCGCTCTCACCTACCAAACAATGCCCCCCTGCAAAAAATAAATTCATAT-
  3' TGCAGATTAAGGCGAGAGTGGATGGTTTGTTACGGGGGGACGTTTTTTATTTAAGTATA-


5    -AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-
     -TTTTTTGTATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAACTGTATTT-


     -TACCACTGGCGGTGATACTGAGCACAT  3'  (SEQ ID NO:53)
     -ATGGTGACCGCCACTATGACTCGTGTAGC5'  (SEQ ID NO:54)
10                              ClaI
```

and then (c) substituting the small DNA sequence between
the unique *ClaI* and *KpnI* restriction sites with the
following oligonucleotide:

15

```
  5' 	CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC3'
     (SEQ ID NO:48)
  3' 	   TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC      5'
20   (SEQ ID NO:49)
       ClaI                                           KpnI
```

The expression plasmid pAMG21 can then be derived from
pCFM1656 by making a series of site directed base
25 changes by PCR overlapping oligo mutagenesis and DNA
sequence substitutions. Starting with the BglII site
(plasmid bp # 180) immediately 5' to the plasmid
replication promoter PcopB and proceeding toward the
plasmid replication genes, the base pair changes are as
30 follows:

pAMG21 bp #	bp in pCFM1656	bp changed to in pAMG21
# 204	T/A	C/G
# 428	A/T	G/C
# 509	G/C	A/T
# 617	- -	insert two G/C bp
# 679	G/C	T/A
# 980	T/A	C/G
# 994	G/C	A/T
# 1004	A/T	C/G

```
          # 1007              C/G                    T/A
          # 1028              A/T                    T/A
          # 1047              C/G                    T/A
          # 1178              G/C                    T/A
    5     # 1466              G/C                    T/A
          # 2028              G/C                    bp deletion
          # 2187              C/G                    T/A
          # 2480              A/T                    T/A

   10   # 2499-2502           AGTG                   GTCA
                              TCAC                   CAGT

        # 2642                TCCGAGC                7 bp deletion
                              AGGCTCG
   15
          # 3435              G/C                    A/T
          # 3446              G/C                    A/T
          # 3643              A/T                    T/A
```

20

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

25

```
[AatII sticky end]                    5'      GCGTAACGTATGCATGGTCTCC-
(position #4358 in pAMG21)             3' TGCACGCATTGCATACGTACCAGAGG-

30  -CCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACT-
    -GGTACGCTCTCATCCCTTGACGGTCCGTAGTTTATTTTGCTTTCCGAGTCAGCTTTCTGA-

    -GGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGC-
35  -CCCGGAAAGCAAAATAGACAACAAACAGCCACTTGCGAGAGGACTCATCCTGTTTAGGCG-

    -CGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGC-
    -GCCCTCGCCTAAACTTGCAACGCTTCGTTGCCGGGCCTCCCACCGCCCGTCCTGCGGGCG-

    -CATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGT-
40  -GTATTTGACGGTCCGTAGTTTAATTCGTCTTCCGGTAGGACTGCCTACCGGAAAAACGCA-

                                                   AatII
    -TTCTACAAACTCTTTTGTTTATTTTTCTAAATACATTCAAATATGGACGTCGTACTTAAC-
    -AAGATGTTTGAGAAAACAAATAAAAAGATTTATGTAAGTTTATACCTGCAGCATGAATTG-
45
    -TTTTAAAGTATGGGCAATCAATTGCTCCTGTTAAAATTGCTTTAGAAATACTTTGGCAGC-
    -AAAATTTCATACCCGTTAGTTAACGAGGACAATTTTAACGAAATCTTTATGAAACCGTCG-

    -GGTTTGTTGTATTGAGTTTCATTTGCGCATTGGTTAAATGGAAAGTGACCGTGCGCTTAC-
50  -CCAAACAACATAACTCAAAGTAAACGCGTAACCAATTTACCTTTCACTGGCACGCGAATG-
```

```
     -TACAGCCTAATATTTTTGAAATATCCCAAGAGCTTTTTCCTTCGCATGCCCACGCTAAAC-
     -ATGTCGGATTATAAAAACTTTATAGGGTTCTCGAAAAAGGAAGCGTACGGGTGCGATTTG-

 5   -ATTCTTTTTCTCTTTTGGTTAAATCGTTGTTTGATTTATTATTTGCTATATTTATTTTTC-
     -TAAGAAAAAGAGAAAACCAATTTAGCAACAAACTAAATAATAAACGATATAAATAAAAAG-

     -GATAATTATCAACTAGAGAAGGAACAATTAATGGTATGTTCATACACGCATGTAAAAATA-
10   -CTATTAATAGTTGATCTCTTCCTTGTTAATTACCATACAAGTATGTGCGTACATTTTTAT-

     -AACTATCTATATAGTTGTCTTTCTCTGAATGTGCAAAACTAAGCATTCCGAAGCCATTAT-
     -TTGATAGATATATCAACAGAAAGAGACTTACACGTTTTGATTCGTAAGGCTTCGGTAATA-

15   -TAGCAGTATGAATAGGGAAACTAAACCCAGTGATAAGACCTGATGATTTCGCTTCTTTAA-
     -ATCGTCATACTTATCCCTTTGATTTGGGTCACTATTCTGGACTACTAAAGCGAAGAAATT-

     -TTACATTTGGAGATTTTTTATTTACAGCATTGTTTTCAAATATATTCCAATTAATCGGTG-
     -AATGTAAACCTCTAAAAAATAAATGTCGTAACAAAAGTTTATATAAGGTTAATTAGCCAC-

20   -AATGATTGGAGTTAGAATAATCTACTATAGGATCATATTTTATTAAATTAGCGTCATCAT-
     -TTACTAACCTCAATCTTATTAGATGATATCCTAGTATAAAATAATTTAATCGCAGTAGTA-

     -AATATTGCCTCCATTTTTTAGGGTAATTATCCAGAATTGAAATATCAGATTTAACCATAG-
25   -TTATAACGGAGGTAAAAAATCCCATTAATAGGTCTTAACTTTATAGTCTAAATTGGTATC-

     -AATGAGGATAAATGATCGCGAGTAAATAATATTCACAATGTACCATTTTAGTCATATCAG-
     -TTACTCCTATTTACTAGCGCTCATTTATTATAAGTGTTACATGGTAAAATCAGTATAGTC-

30   -ATAAGCATTGATTAATATCATTATTGCTTCTACAGGCTTTAATTTTATTAATTATTCTGT-
     -TATTCGTAACTAATTATAGTAATAACGAAGATGTCCGAAATTAAAATAATTAATAAGACA-

     -AAGTGTCGTCGGCATTTATGTCTTTCATACCCATCTCTTTATCCTTACCTATTGTTTGTC-
     -TTCACAGCAGCCGTAAATACAGAAAGTATGGGTAGAGAAATAGGAATGGATAACAAACAG-

35   -GCAAGTTTTGCGTGTTATATATCATTAAAACGGTAATAGATTGACATTTGATTCTAATAA-
     -CGTTCAAAACGCACAATATATAGTAATTTTGCCATTATCTAACTGTAAACTAAGATTATT-

     -ATTGGATTTTTGTCACACTATTATATCGCTTGAAATACAATTGTTTAACATAAGTACCTG-
40   -TAACCTAAAAACAGTGTGATAATATAGCGAACTTTATGTTAACAAATTGTATTCATGGAC-

     -TAGGATCGTACAGGTTTACGCAAGAAATGGTTTGTTATAGTCGATTAATCGATTTGATT-
     -ATCCTAGCATGTCCAAATGCGTTCTTTTACCAAACAATATCAGCTAATTAGCTAAACTAA-

     -CTAGATTTGTTTTAACTAATTAAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGA-
45   -GATCTAAACAAAATTGATTAATTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGCT-

                                                        SacII
     -GCTCACTAGTGTCGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGGAAAGAA-
50   -CGAGTGATCACAGCTGGACGTCCCATGGTACCTTCGAATGAGCTCCTAGGCGCCTTTCTT-

     -GAAGAAGAAGAAGAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA-
     -CTTCTTCTTCTTCTTTCGGGCTTTCCTTCGACTCAACCGACGACGGTGGCGACTCGTTAT-

55   -ACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGG-
     -TGATCGTATTGGGGAACCCCGGAGATTTGCCCAGAACTCCCCAAAAAACGACTTTCCTCC-
```

- 75 -

-AACCGCTCTTCACGCTCTTCACGC 3' [SacII sticky end] (SEQ ID NO:50)
-TTGGCGAGAAGTGCGAGAAGTG   5' (position #5904 in pAMG21) (SEQ ID
NO:46)

5

During the ligation of the sticky ends of this
substitution DNA sequence, the outside AatII and SacII
sites are destroyed. There are unique AatII and SacII
sites in the substituted DNA.

10

pAMG22-His

The expression plasmid pAMG22-His can be derived from
the Amgen expression vector pAMG22 by substituting the
small DNA sequence between the unique NdeI (#4795) and
15   EcoRI (#4818) restriction sites of pAMG22 with the
following oligonucleotide duplex:

```
     NdeI                                    NheI        EcoRI
     5'  TATGAAACATCATCACCATCACCATCATGCTAGCGTTAACGCGTTGG      3'
20   (SEQ ID NO:51)

     3'      ACTTTGTAGTAGTGGTAGTGGTAGTACGATCGCAATTGCGCAACCTTAA   5'
     (SEQ ID NO:52)

         MetLysHisHisHisHisHisHisHisAlaSerValAsnAlaLeuGlu
     (SEQ ID NO:168)
```

25

pAMG22

The expression plasmid pAMG22 can be derived from the
Amgen expression vector pCFM1656 (ATCC #69576) which in
turn be derived from the Amgen expression vector system
30   described in US Patent No. 4,710,473 granted December 1,
1987. The pCFM1656 plasmid can be derived from the
described pCFM836 plasmid (Patent No. 4,710,473) by:
(a) destroying the two endogenous NdeI restriction
sites by end filling with T4 polymerase enzyme followed
35   by blunt end ligation; (b) replacing the DNA sequence
between the unique AatII and ClaI restriction sites

- 76 -

containing the synthetic PL promoter with a similar
fragment obtained from pCFM636 (patent No. 4,710,473)
containing the PL promoter

5   AatII

5'   CTAATTCCGCTCTCACCTACCAAACAATGCCCCCCTGCAAAAAATAAATTCATAT-
3' TGCAGATTAAGGCGAGAGTGGATGGTTTGTTACGGGGGGACGTTTTTTATTTAAGTATA-

-AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-
10 -TTTTTTGTATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAACTGTATTT-

-TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)
-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)
                        ClaI
15

and then (c) substituting the small DNA sequence between
the unique ClaI and KpnI restriction sites with the
following oligonucleotide:

20   5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC 3'
     (SEQ ID NO:55)
     3'   TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC   5'
     (SEQ ID NO:56)
        ClaI
                                                    KpnI
25

The expression plasmid pAMG22 can then be derived from
pCFM1656 by making a series of site directed base
changes by PCR overlapping oligo mutagenesis and DNA
sequence substitutions. Starting with the BglII site
30 (plasmid bp # 180) immediately 5' to the plasmid
replication promoter PcopB and proceeding toward the
plasmid replication genes, the base pair changes are as
follows:

- 77 -

	pAMG22 bp #	bp in pCFM1656	bp changed to in pAMG22
5	# 204	T/A	C/G
	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
10	# 679	G/C	T/A
	# 980	T/A	C/G
	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
15	# 1028	A/T	T/A
	# 1047	C/G	T/A
	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
20	# 2187	C/G	T/A
	# 2480	A/T	T/A
	# 2499-2502	AGTG	GTCA
25		TCAC	CAGT
	# 2642	TCCGAGC	7 bp deletion
		AGGCTCG	
	# 3435	G/C	A/T
30	# 3446	G/C	A/T
	# 3643	A/T	T/A

The DNA sequence between the unique AatII (position
#4364 in pCFM1656) and *SacII* (position #4585 in
35 pCFM1656) restriction sites is substituted with the
following DNA sequence:

[AatII sticky end] (position #4358 in pAMG22)

```
40  5'    GCGTAACGTATGCATGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAA-
    3' TGCACGCATTGCATACGTACCAGAGGGGTACGCTCTCATCCCTTGACGGTCCGTAGTT-

    -ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTG-
    -TATTTTGCTTTCCGAGTCAGCTTTCTGACCCGGAAAGCAAAATAGACAACAAACAGCCAC-
45
```

- 78 -

```
-AACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGG-
-TTGCGAGAGGACTCATCCTGTTTAGGCGGCCCTCGCCTAAACTTGCAACGCTTCGTTGCC-
```

5
```
-CCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAG-
-GGGCCTCCCACCGCCCGTCCTGCGGGCGGTATTTGACGGTCCGTAGTTTAATTCGTCTTC-
```

```
-GCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTGTTTATTTTTCTAAAT-
-CGGTAGGACTGCCTACCGGAAAAACGCAAAGATGTTTGAGAAAACAAATAAAAAGATTTA-
```

10   AatII
```
-ACATTCAAATATGGACGTCTCATAATTTTTAAAAAATTCATTTGACAAATGCTAAAATTC-
-TGTAAGTTTATACCTGCAGAGTATTAAAAATTTTTTAAGTAAACTGTTTACGATTTTAAG-
```

```
-TTGATTAATATTCTCAATTGTGAGCGCTCACAATTTATCGATTTGATTCTAGATTTGTTT-
15 -AACTAATTATAAGAGTTAACACTCGCGAGTGTTAAATAGCTAAACTAAGATCTAAACTCA-
```

```
-TAACTAATTAAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGAGCTCACTAGTGT-
-ATTGATTAATTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGCTCGAGTGATCACA-
```

20   SacII
```
-CGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGGAAAGAAGAAGAAGAAGAA-
-GCTGGACGTCCCATGGTACCTTCGAATGAGCTCCTAGGCGCCTTTCTTCTTCTTCTTCTT-
```

```
-GAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC-
25 -CTTTCGGGCTTTCCTTCGACTCAACCGACGACGGTGGCGACTCGTTATTGATCGTATTGG-
```

```
-CCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACCGCTCTTCA-
-GGAACCCCGGAGATTTGCCCAGAACTCCCCAAAAAACGACTTTCCTCCTTGGCGAGAAGT-
```

30 -CGCTCTTCACGC 3' (SEQ ID NO:58)
    -GCGAGAAGTG   5' (SEQ ID NO:57)

        [SacII sticky end] (position #5024 in pAMG22)

35 During the ligation of the sticky ends of this
    substitution DNA sequence, the outside AatII and SacII
    sites are destroyed. There are unique AatII and SacII
    sites in the substituted DNA.

40 B. Human OPG Met [32-401]

            In the example, the expression vector used was
    pAMG21, a derivative of pCFM1656 (ATCC accession no.
    69576) which contains appropriate restriction sites for
    insertion of genes downstream from the lux PR promoter.
45 (See U.S. Patent No. 5,169,318 for description of the
    lux expression system). The host cell used was GM120
```

- 79 -

(ATCC accession no. 55764). This host has the lacIQ promoter and lacI gene integrated into a second site in the host chromosome of a prototrophic E. coli K12 host. Other commonly used E. coli expression vectors and host
5  cells are also suitable for expression.

A DNA sequence coding for an N-terminal methionine and amino acids 32-401 of the human OPG polypeptide was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as
10  follows. To accomplish this, PCR using oligonucleotides #1257-20 and #1257-19 as primers was performed using as a template plasmid pRcCMV-Hu OPG DNA containing the human OPG cDNA and thermocycling for 30 cycles with each cycle being: 94°C for 20 seconds, followed by 37°C for
15  30 seconds, followed by 72°C for 30 seconds. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, and restricted with KpnI and BamHI restriction endonucleases and purified. Synthetic oligonucleotides #1257-21 and #1257-22 were
20  phophorylated individually using T4 polynucleotide kinase and ATP, and were then mixed together, heated at 94°C and allowed to slow cool to room temperature to form an oligonucleotide linker duplex containing NdeI and KpnI sticky ends. The phosphorylated linker duplex
25  formed between oligonucleotides #1257-21 and #1257-22 containing NdeI and KpnI cohesive ends (see Figure 14A) and the KpnI and BamHI digested and purified PCR product generated using oligo primers #1257-20 and #1257-19 (see above) was directionally inserted between two sites of
30  the plasmid vector pAMG21, namely the NdeI site and BamHI site, using standard recombinant DNA methodology (see Figure 14A and sequences below). The synthetic linker utilized E. coli codons and provided for a N-terminal methionine.

- 80 -

Two clones were selected and plasmid DNA isolated, and the human OPG insert was subsequently DNA sequence confirmed. The resulting pAMG21 plasmid containing amino acids 32-401 of the human OPG
5      polypeptide immediately preceded in frame by a methionine is referred to as pAMG21-huOPG met[32-401] or pAMG21-huOPG met[32-401].


       Oligo#1257-19
10     5'-TACGCACTGGATCCTTATAAGCAGCTTATTTTTACTGATTGGAC-3'
       (SEQ ID NO:59)


       Oligo#1257-20
       5'-GTCCTCCTGGTACCTACCTAAAACAAC-3'  (SEQ ID NO:60)
15

       Oligo#1257-21
       5'-TATGGATGAAGAAACTTCTCATCAGCTGCTGTGTGATAAATGTCC
            GCCGGGTAC -3'  (SEQ ID NO:61)


20   ·Oligo#1257-22
       5'-CCGGCGGACATTTATCACACAGCAGCTGATGAGAAGTTTCTTCATCCA-3'
       (SEQ ID NO:47)


                Cultures of pAMG21-huOPG met[32-401] in E.
25   coli GM120 in 2XYT media containing 20 µg/ml kanamycin
       were incubated at 30°C prior to induction. Induction of
       huOPG met[32-401] gene product expression from the luxPR
       promoter was achieved following the addition of the
       synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine
30   lactone to the culture media to a final concentration of
       30 ng/ml and cultures were incubated at either 30°C or
       37°C for a further 6 hours. After 6 hours, the
       bacterial cultures were examined by microscopy for the
       presence of inclusion bodies and were then pelletted by

centrifugation.  Refractile inclusion bodies were observed in induced cultures indicating that some of the recombinant huOPG met[32-401] gene product was produced insolubly in E. coli.  Some bacterial pellets were

5    resuspended in 10mM Tris-HCl/pH8, 1mM EDTA and lysed directly by addition of 2X Laemlli sample buffer to 1X final, and β-mercaptoethanol to 5% final concentration, and analyzed by SDS-PAGE.  A substantially more intense coomassie stained band of approximately 42kDa was

10   observed on a SDS-PAGE gel containing total cell lysates of 30°C and 37°C induced cultures versus lane 2 which is a total cell lysate of a 30°C uninduced culture (Figure 14B).  The expected gene product would be 370 amino acids in length and have an expected molecular weight of

15   about 42.2 kDa.  Following induction at 37°C for 6 hours, an additional culture was pelleted and either processed for isolation of inclusion bodies (see below) or processed by microfluidizing.  The pellet processed for microfluidizing was resuspended in 25mM Tris-

20   HCl/pH8, 0.5M NaCl buffer and passed 20 times through a Microfluidizer Model 1108 (Microfluidics Corp.) and collected.  An aliquot was removed of the collected sample (microfluidized total lysate), and the remainder was pelleted at 20,000 x g for 20 minutes.  The

25   supernatant following centrifugation was removed (microfluidized soluble fraction) and the pellet resuspended in a 25mM Tris-HCl/pH8, 0.5M NaCl, 6M urea solution (microfluidized insoluble fraction).  To an aliquot of either the total soluble, or insoluble

30   fraction was added to an equal volume of 2X Laemalli sample buffer and β-mercaptoethanol to 5% final concentration.  The samples were then analyzed by SDS-PAGE.  A significant amount of recombinant huOPG met[32-401] gene product appeared to be found in the

- 82 -

insoluble fraction.  To purify the recombinant protein inclusion bodies were purified as follows: Bacterial cells were separated from media by density gradient centrifugation in a Beckman J-6B centrifuge equipped
5   with a JS-4.2 rotor at 4,900 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of water and then diluted to a final volume of 10 ml with water. This suspension was transferred to a stainless steel cup cooled in ice and subjected to sonic disruption using a
10  Branson Sonifier equipped with a standard tip (power setting=5, duty cycle=95%, 80 bursts).  The sonicated cell suspension was centrifuged in a Beckman Optima TLX ultracentrifuge equipped with a TLA 100.3 rotor at 195,000 x g for 5 to 10 minutes at 23°C.  The
15  supernatant was discarded and the pellet rinsed with a stream of water from a squirt bottle.  The pellets were collected by scraping with a micro spatula and transferred to a glass homogenizer (15 ml capacity). Five ml of Percoll solution (75% liquid Percoll, 0.15 M
20  sodium chloride) was added to the homogenizer and the contents are homogenized until uniformly suspended.  The volume was increased to 19.5 ml by the addition of Percoll solution, mixed, and distributed into 3 Beckman Quick-Seal tubes (13 x 32 mm).  Tubes were sealed
25  according to manufacturers instructions.  The tubes were spun in a Beckman TLA 100.3 rotor at 23°C, 20,000 rpm (21,600 x g), 30 minutes.  The tubes were examined for the appropriate banding pattern.  To recover the refractile bodies, gradient fractions were recovered and
30  pooled, then diluted with water.  The inclusion bodies were pelleted by centrifugation, and the protein concentration estimated following SDS-PAGE.

An aliquot of inclusion bodies isolated as described below was dissolved into 1X Laemlli sample

- 83 -

buffer with 5% β-mercaptoethanol and resolved on a SDS-
PAGE gel and the isolated inclusion bodies provide a
highly purified recombinant huOPG[32-401] gene product.
The major ~42 kDa band observed after resolving
5    inclusion bodies on a SDS-polyacrylamide gel was excised
from a separate gel and the N-terminal amino acid
sequence determined essentially as described (Matsudaira
et al. J. Biol. Chem. 262, 10-35 (1987)).  The following
sequence was determined after 19 cycles:

10

        NH2 -MDEETSHQLLCDKCPPGTY-COOH  (SEQ ID NO:62)


This sequence was found to be identical to the first 19
amino acids encoded by the pAMG21 Hu-OPG met[32-401]
15   expression vector, produced by a methionine residue
provided by the bacterial expression vector.

C. Human OPG met[22-401]

        A DNA sequence coding for an N-terminal
20   methionine and amino acids 22 through 401 of human OPG
was placed under control of the luxPR promoter in a
prokaryotic plasmid expression vector pAMG21 as follows.
Isolated plasmid DNA of pAMG21-huOPG met[32-401] (see
Section B) was cleaved with KpnI and BamHI restriction
25   endonucleases and the resulting fragments were resolved
on an agarose gel.  The B fragment (~1064 bp fragment)
was isolated from the gel using standard methodology.
Synthetic oligonucleotides (oligos) #1267-06 and #1267-
07 were phosphorylated individually and allowed to form
30   an oligo linker duplex, which contained NdeI and KpnI
cohesive ends, using methods described in Section B.
The synthetic linker duplex utilized E. coli codons and
provided for an N-terminal methionine.  The
phosphorylated oligo linker containing NdeI and KpnI

- 84 -

cohesive ends and the isolated ~1064 bp fragment of
pAMG21-huOP met[32-401] digested with KpnI and BamHI
restriction endonucleases were directionally inserted
between the NdeI and BamHI sites of pAMG21 using

5    standard recombinant DNA methodology.  The ligation
mixture was transformed into E. coli host 393 by
electroporation utilizing the manufacturer's protocol.
Clones were selected, plasmid DNA was isolated, and DNA
sequencing was performed to verify the DNA sequence of

10   the huOPG-met[22-401] gene.


     Oligo #1267-06
5'-TAT GGA AAC TTT TCC TCC AAA ATA TCT TCA TTA TGA TGA
AGA AAC TTC TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC GGG

15   TAC-3' (SEQ ID NO:63)


     Oligo #1267-07
5'-CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAG AAG TTT
CTT  CAT CAT AAT GAA GAT ATT TTG GAG GAA AAG TTT CCA-3'

20   (SEQ ID NO:64)


          Cultures of pAMG21-huOPG-met[22-401] in E.
coli host 393 were placed in 2XYT media containing 20
μg/ml kanamycin and were incubated at 30°C prior to

25   induction.  Induction of recombinant gene product
expression from the luxPR promoter of vector pAMG21 was
achieved following the addition of the synthetic
autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to
the culture media to a final concentration of 30 ng/ml

30   and incubation at either 30°C or 37°C for a further 6
hours.  After 6 hours, bacterial cultures were pelleted
by centrifugation (=30°C I+6 or 37°C I+6).  Bacterial
cultures were also either pelleted just prior to
induction (=30°C PreI) or alternatively no autoinducer

- 85 -

was added to a separate culture which was allowed to
incubate at 30°C for a further 6 hours to give an
uninduced (UI) culture (=30°C UI).  Bacterial pellets of
either 30°C PreI, 30°C UI, 30°C I+6, or 37°C I+6
5    cultures were resuspended, lysed, and analyzed by SDS-
polyacrylamide gel electrophoresis (PAGE) as described
in Section B.  Polyacrylamide gels were either stained
with coomassie blue and/or Western transferred to
nitrocellulose and immunoprobed with rabbit anti-mu
10   OPG-Fc polyclonal antibody as described in Example 10.
The level of gene product following induction compared
to either an uninduced (30°C UI) or pre-induction (30°C
PreI) sample.


15   D. Murine OPG met [22-401]

A DNA sequence coding for an N-terminal
methionine and amino acids 22 through 401 of the murine
(mu) OPG (OPG) polypeptide was placed under control of
the luxPR promoter in a prokaryotic plasmid expression
20   vector pAMG21 as follows.  PCR was performed using
oligonucleotides #1257-16 and #1257-15 as primers,
plasmid pRcCMV-Mu OPG DNA as a template and
thermocycling conditions as described in Section B.  The
PCR product was purified and cleaved with KpnI and BamHI
25   restriction endonucleases as described in Section B.
Synthetic oligos #1260-61 and #1260-82 were
phosphorylated individually and allowed to form an oligo
linker duplex with NdeI and KpnI cohesive ends using
methods described in Section B.  The synthetic linker
30   duplex utilized E. coli codons and provided for an N-
terminal methionine.  The phosphorylated linker duplex
formed between oligos #1260-61 and #1260-82 containing
NdeI and KpnI cohesive ends and the KpnI and BamHI
digested and purified PCR product generated using oligo

- 86 -

primers #1257-16 and #1257-15 were directionally
inserted between the NdeI and BamHI sites of pAMG21
using standard methodology.  The ligation mixture was
transformed into E. coli host 393 by electroporation
5   utilizing the manufacturer's protocol.  Clones were
selected, plasmid DNA was isolated, and DNA sequencing
was performed to verify the DNA sequence of the MuOPG
met[22-401] gene.

        Expression of recombinant muOPG met[22-401]
10  polypeptide from cultures of 393 cells harboring plasmid
pAMG21-MuOPG met[22-401] following induction was
determined using methods described in Section C.


        Oligo #1257-15
15      5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTC ACG
GAT   TGA AC-3' (SEQ ID NO:65)


        Oligo #1257-16
        5'-GTG CTC CTG GTA CCT ACC TAA AAC AGC ACT GCA CAG
20  TG-3' (SEQ ID NO:66)


        Oligo #1260-61
        5'-TAT GGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA
TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC
25  GGG TAC-3' (SEQ ID NO:67)


        Oligo #1260-82
        5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CCA-
30  3' (SEQ ID NO:68)


E. Murine OPG met[32-401]

        A DNA sequence coding for an N-terminal
methionine and amino acids 32 through 401 of murine OPG

- 87 -

was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. To accomplish this, Synthetic oligos #1267-08 and #1267-09 were phosphorylated individually and allowed to form

5      an oligo linker duplex using methods described in Section B.  The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine.  The phosphorylated linker duplex formed between oligos #1267-08 and #1267-09 containing NdeI and KpnI cohesive

10     ends, and the KpnI and BamHI digested and purified PCR product described earlier (see Section D), was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology.  The ligation mixture was transformed into E. coli host 393 by

15     electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[32-401] gene.

       Expression of recombinant muOPG-met [32-401]

20     polypeptide from cultures of 393 cells harboring the pAMG21 recombinant plasmid following induction was determined using methods described in Section C.


       Oligo #1267-08

25     5'-TAT GGA CCC AGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC GGG TAC-3' (SEQ ID NO:69)


       Oligo #1267-09

       5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG

30     TTT CTG GGT CCA-3' (SEQ ID NO:70)

- 88 -

E. Murine OPG met-lys[22-401]

A DNA sequence coding for an N-terminal
methionine followed by a lysine residue and amino acids
22 through 401 of murine OPG was placed under control of
5       the lux PR promoter in prokaryotic expression vector
pAMG21 as follows.  Synthetic oligos #1282-95 and #1282-
96 were phosphorylated individually and allowed to form
an oligo linker duplex using methods described in
Section B.  The synthetic linker duplex utilized E. coli
10      codons and provided for an N-terminal methionine.  The
phosphorylated linker duplex formed between oligos
#1282-95 and #1282-96 containing NdeI and KpnI cohesive
ends and the KpnI and BamHI digested and purified PCR
product described in Section D was directionally
15      inserted between the NdeI and BamHI sites in pAMG21
using standard methodology.  The ligation mixture was
transformed into E. coli host 393 by electroporation
utilizing the manufacturer's protocol.  Clones were
selected, plasmid DNA was isolated, and DNA sequencing
20      was performed to verify the DNA sequence of the MuOPG--
Met-Lys[22-401] gene.

Expression of recombinant MuOPG Met-Lys[22-
401] polypeptide from transformed 393 cells harboring
the recombinant pAMG21 plasmid following induction was
25      determined using methods described in Section C.

Oligo #1282-95

5'-TAT GAA AGA AAC TCT GCC TCC AAA ATA CCT GCA TTA
CGA TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC
30      TCC GGG TAC-3'  (SEQ ID NO:71)

- 89 -

Oligo #1282-96

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT
TCA-3' (SEQ ID NO:72)

5

G. Murine OPG met-lys-(his)₇[22-401]

A DNA sequence coding for N-terminal residues
Met-Lys-His-His-His-His-His-His-His (=MKH) followed by
amino acids 22 through 401 of Murine OPG was placed
10    under control of the lux PR promoter in prokaryotic
expression vector pAMG21 as follows.  PCR was performed
using oligonucleotides #1300-50 and #1257-15 as primers
and plasmid pAMG21-muOPG-met[22-401] DNA as template.
Thermocycling conditions were as described in Section B.
15    The resulting PCR sample was resolved on an agarose gel,
the PCR product was excised, purified, cleaved with NdeI
and BamHI restriction endonucleases and purified.  The
NdeI and BamHI digested and purified PCR product
generated using oligo primers #1300-50 and #1257-15 was
20    directionally inserted between the NdeI and BamHI sites
of pAMG21 using standard DNA methodology.  The ligation
mixture was transformed into E. coli host 393 by
electroporation utilizing the manufacturer's protocol.
Clones were selected, plasmid DNA was isolated, and DNA
25    sequencing performed to verify the DNA sequence of the
muOPG-MKH[22-401] gene.

Expression of recombinant MuOPG-MKH[22-401]
polypeptide from transformed 393 cultures harboring the
recombinant pAMG21 plasmid following induction was
30    determined using methods described in Section C.

- 90 -

Oligo #1300-50

5'-GTT CTC CTC ATA TGA AAC ATC ATC ACC ATC ACC ATC ATG AAA CTC TGC CTC CAA AAT ACC TGC ATT ACG AT-3' (SEQ ID NO:73)

5

Oligo #1257-15

(see Section D)

H. Murine OPG met-lys[22-401](his)7

10       A DNA sequence coding for a N-terminal met-lys, amino acids 22 through 401 murine OPG, and seven histidine residues following amino acid 401 (=muOPG MK[22-401]-H7), was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as 15 follows. PCR was performed using oligonucleotides #1300-49 and #1300-51 as primers and pAMG21-muOPG met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product 20 was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation was 25 transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG MK[22-401]-H7 gene.

30       Expression of the recombinant muOPG MK-[22-401]-H7 polypeptide from a transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

- 91 -

Oligo #1300-49

5'-GTT CTC CTC ATA TGA AAG AAA CTC TGC CTC CAA AAT ACC TGC A-3' (SEQ ID NO:74)

5

Oligo #1300-51

5'-TAC GCA CTG GAT CCT TAA TGA TGG TGA TGG TGA TGA TGT AAG CAG CTT ATT TTC ACG GAT TGA ACC TGA TTC CCT A-3' (SEQ ID NO:75)

10

I. Murine OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of murine OPG was placed under control of the lux PR promoter of

15 prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-74 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was

20 resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites of pAMG21

25 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the

30 muOPG-met[27-401] gene.

Expression of recombinant muOPG-met[27-401] polypeptide from a transfected 393 culture harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

- 92 -

Oligo#1309-74

5'-GTT CTC CTC ATA TGA AAT ACC TGC ATT ACG ATC CGG AAA CTG GTC AT-3' (SEQ ID NO:76)

5

Oligo#1257-15

(See Section D)


J. Human OPG met[27-401]

10            A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of human OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows.  PCR was performed using oligonucleotides #1309-75 and #1309-

15     76 as primers and plasmid pAMG21-huOPG-met[22-401] DNA as template.  Thermocycling conditions were as described in Section B.  The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with AseI and BamHI restriction

20     endonucleases, and purified.  The AseI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology.  The ligation mixture was transformed into E. coli host 393 by

25     electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[27-401] gene.

            Expression of the recombinant huOPG-met[27-

30     401] polypeptide following induction of from transfected 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

- 93 -

        Oligo #1309-75

        5'-GTT CTC CTA TTA ATG AAA TAT CTT CAT TAT GAT GAA
GAA ACT T-3'  (SEQ ID NO:77)

5

        Oligo #1309-76

        5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTT ACT
GAT T-3'  (SEQ ID NO:78)

10      K. Murine OPG met[22-180]

        A DNA sequence coding for a N-terminal
methionine and amino acids 22 through 180 of murine OPG
was placed under control of the lux PR promoter of
prokaryotic expression vector pAMG21 as follows.  PCR
15      was performed with oligonucleotides #1309-72 and #1309-
73 as primers and plasmid pAMG21-muOPG-met[22-401] DNA
as template.  Thermocycling conditions were as described
in Section B.  The resulting PCR sample was resolved on
an agarose gel, the PCR product was excised, purified,
20      restricted with NdeI and BamHI restriction
endonucleases, and purified.  The NdeI and BamHI
digested and purified PCR product above was
directionally inserted between the NdeI and BamHI sites
of pAMG21 using standard methodology.  The ligation was
25      transformed into E. coli  host 393 by electroporation
utilizing the manufacturer's protocol.  Clones were
selected, plasmid DNA was isolated, and DNA sequencing
was performed to verify the DNA sequence of the muOPG-
met[22-180] gene.
30              Expression of recombinant muOPG-met[22-180]
polypeptide from transformed 393 cultures harboring the
recombinant pAMG21 plasmid following induction was
determined using methods described in Section C.

- 94 -

Oligo #1309-72

5'-GTT CTC CTC ATA TGG AAA CTC TGC CTC CAA AAT ACC TGC A-3' (SEQ ID NO:79)

5      Oligo #1309-73

5'-TAC GCA CTG GAT CCT TAT GTT GCA TTT CCT TTC TGA ATT AGC A-3' (SEQ ID NO:80)

L. Murine OPG met [27-180]

10          A DNA sequence coding for a N-terminal methionine and amino acids 27 through 180 of murine OPG was placed under the control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-74 (see

15   Section I) and #1309-73 (see Section K) as primers and plasmid pAMG21-muOPG met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product excised, purified, restricted with NdeI

20   and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393

25   by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG met[27-180] gene.

          Expression of recombinant muOPG met[27-180]
30   polypeptide from cultures of transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

- 95 -

M. Murine OPG met[22-189] and met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 189, or 22 through 194 of murine OPG was placed under control of
5  the lux PR promoter of prokaryotic expression vector pAMG21 as follows.  The pair of synthetic oligonucleotides #1337-92 and #1337-93 (=muOPG-189 linker) or #1333-57 and #1333-58 (=muOPG-194 linker) were phosphorylated individually and allowed to form an
10 oligo linker duplex pair using methods described in Section B.  Purified plasmid DNA of pAMG21-muOPG-met[22-401] was cleaved with KpnI and BspEI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~413 bp B fragment was
15 isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1337-92 and #1337-93 (muOPG-189 linker) or oligos #1333-57 and #1333-58 (muOPG-194 linker) containing BspEI and BamHI cohesive ends, and the
20 isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401]  digested with KpnI and BspEI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of pAMG21-muOPG met[22-401] using standard methodology.  Each ligation mixture was
25 transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol.  Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the muOPG-met[22-189] or muOPG-met[22-194] genes.
30          Expression of recombinant muOPG-met[22-189] and muOPG-met[22-194] polypeptides from recombinant pAMG21 plasmids transformed into 393 cells was determined using methods described in Section C.

- 96 -

Oligo #1337-92

5'-CCG GAA ACA GAT AAT GAG-3' (SEQ ID NO:81)


Oligo #1337-93

5   5'-GAT CCT CAT TAT CTG TTT-3' (SEQ ID NO:82)


Oligo #1333-57

5'-CCG GAA ACA GAG AAG CCA CGC AAA AGT AAG-3'
(SEQ ID NO:83)

10

Oligo #1333-58

5'-GAT CCT TAC TTT TGC GTG GCT TCT CTG TTT-3'
(SEQ ID NO:84)


15   N. Murine OPG met[27-189] and met[27-194]

A DNA sequence coding for a N-terminal
methionine and either amino acids 27 through 189, or 27
through 194 of murine OPG was placed under control of
the lux PR promoter of prokaryotic expression vector
20   pAMG21 as follows. Phosphorylated oligo linkers either
"muOPG-189 linker" or "muOPG-194 linker" (see Section M)
containing BspEI and BamHI cohesive ends, and the
isolated ~413 bp B fragment of plasmid pAMG21-muOPG-
met[22-401] digested with KpnI and BspEI restriction
25   endonucleases were directionally inserted between the
KpnI and BamHI sites of plasmid pAMG21-muOPG-met[27-401]
using standard methodology. Each ligation was
transformed into E. coli host 393 by electroporation
utilizing the manufacturer's protocol. Clones were
30   selected, plasmid DNA was isolated, and DNA sequencing
was performed to verify the DNA sequence of either the
muOPG met[27-189] or muOPG met[27-194] genes.

Expression of recombinant muOPG met[27-189]
and muOPG met[27-194] following induction of 393 cells

- 97 -

harboring recombinant pAMG21 plasmids was determined
using methods described in Section C.

O. Human OPG met[22-185], met[22-189], met[22-194]

5          A DNA sequence coding for a N-terminal
methionine and either amino acids 22 through 185, 22
through 189, or 22 through 194 of the human OPG
polypeptide was placed under control of the lux PR
promoter of prokaryotic expression vector pAMG21 as
10    follows.  The pair of synthetic oligonucleotides #1331-
87 and #1331-88 (=huOPG-185 linker), #1331-89 and #1331-
90 (=huOPG-189 linker), or #1331-91 & #1331-92 (=huOPG-
194 linker) were phosphorylated individually and each
allowed to form an oligo linker duplex pair using
15    methods described in Section B.  Purified plasmid DNA of
pAMG21-huOPG-met[27-401] was restricted with KpnI and
NdeI restriction endonucleases and the resulting DNA
fragments were resolved on an agarose gel.  The ~407 bp
B fragment was isolated using standard recombinant DNA
20    methodology.  The phophorylated oligo linker duplexes
formed between either oligos #1331-87 and #1331-88
(huOPG-185 linker), oligos #1331-89 and #1331-90 (huOPG-
189 linker), or oligos #1331-91 and #1331-92  (huOPG-194
linker)[each linker contains NdeI and BamHI cohesive
25    ends], and the isolated ~407 bp B fragment of plasmid
pAMG21-huOPG-met[27-401] digested with KpnI and NdeI
restriction endonucleases above, was directionally
inserted between the KpnI and BamHI sites of plasmid
pAMG21-huOPG-met[22-401] using standard methodology.
30    Each ligation was transformed into E. coli host 393 by
electroporation utilizing the manufacturer's protocol.
Clones were selected, plasmid DNA was isolated, and DNA
sequencing was performed to verify the DNA sequence of

- 98 -

either the huOPG-met[22-185], huOPG-met[22-189], or
huOPG-met[22-194] genes.

Expression of recombinant huOPG-met[22-185],
huOPG-met[22-189] or huOPG-met[22-194] in transformed
5    393 cells harboring recombinant pAMG21 plasmids
following induction was determined using methods
described in Section C.


         Oligo #1331-87
10       5'-TAT GTT AAT GAG-3' (SEQ ID NO:85)


         Oligo #1331-88
         5'-GAT CCT CAT TAA CA-3' (SEQ ID NO:86)


15       Oligo #1331-89
         5'-TAT GTT CCG GAA ACA GTT AAG-3' (SEQ ID NO:87)


         Oligo #1331-90
         5'-GAT CCT TAA CTG TTT CCG GAA CA-3' (SEQ ID NO:88)
20

         Oligo #1331-91
         5'-TAT GTT CCG GAA ACA GTG AAT CAA CTC AAA AAT
AAG-3' (SEQ ID NO:89)


25       Oligo #1331-92
         5'-GAT CCT TAT TTT TGA GTT GAT TCA CTG TTT CCG GAA
CA-3' (SEQ ID NO:90)


30  P. Human OPG met[27-185], met[27-189], met [27-194]

A DNA sequence coding for a N-terminal
methionine and either amino acids 27 through 185, 27
through 189, or 27 through 194 of the human OPG
polypeptide was placed under control of the lux PR

- 99 -

promoter of prokaryotic expression vector pAMG21 as
follows.  Phosphorylated oligo linkers "huOPG-185
linker", "huOPG-189 linker", or "huOPG-194 linker" (See
Section O) each containing NdeI and BamHI cohesive ends,
5    and the isolated ~407 bp B fragment of plasmid pAMG21-
huOPG-met[27-401] digested with KpnI and NdeI
restriction endonucleases (See Section O) were
directionally inserted between the KpnI and BamHI sites
of plasmid pAMG21-huOPG-met[27-401] (See Section J)
10   using standard methodology.  Each ligation was
transformed into E. coli host 393 by electroporation
utilizing the manufacturer's protocol.  Clones were
selected, plasmid DNA isolated, and DNA sequencing
performed to verify the DNA sequence of either the
15   huOPG-met[27-185], huOPG-met[27-189], or huOPG-met[27-
194] genes.

          Expression of recombinant huOPG-met[27-185],
huOPG-met[27-189], and huOPG-met[27-194] from
recombinant pAMG21 plasmids transformed into 393 cells
20   was determined using methods described in Section C.


       O. Murine OPG met[27-401] (P33E, G36S, A45P)

          A DNA sequence coding for an N-terminal
25   methionine and amino acids 27 through 48 of human OPG
followed by amino acid residues 49 through 401 of murine
OPG was placed under control of the lux PR promoter of
prokaryotic expression vector pAMG21 as follows.
Purified plasmid DNA of pAMG21-huOPG-met[27-401] (See
30   Section J) was cleaved with AatII and KpnI restriction
endonucleases and a ~1075 bp B fragment isolated from an
agarose gel using standard recombinant DNA methodology.
Additionally, plasmid pAMG21-muOPG-met[22-401] DNA (See
Section D) was digested with KpnI and BamHI restriction

- 100 -

endonucleases and the ~1064 bp B fragment isolated as described above.  The isolated ~1075 bp pAMG21-huOPG-met[27-401] restriction fragment containing AatII & KpnI cohesive ends (see above), the ~1064 bp pAMG21-muOPG-
5    met[22-401] restriction fragment containing KpnI and BamHI sticky ends and a  ~5043 bp restriction fragment containing AatII and BamHI cohesive ends and corresponding to the nucleic acid sequence of pAMG21 between  AatII & BamHI were ligated using standard
10   recombinant DNA methodology.  The ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol.  Clones were selected, and the presence of the recombinant insert in the plasmid verified using standard DNA methodology.
15   muOPG-27-401 (P33E, G36S, A45P) gene.  Amino acid changes in muOPG from proline-33 to glutamic acid-33, glycine-36 to serine-36, and alanine-45 to proline-45, result from replacement of muOPG residues 27 through 48 with huOPG residues 27 through 48.
20        Expression of recombinant muOPG-met[27-401] (P33E, G36S, A45P) from transformed 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.


25

R. Murine OPG met-lys-(his)7-ala-ser-(asp)4-lys[22-401] (A45T)

     A DNA sequence coding for an N-terminal His tag and enterokinase recognition sequence which is (NH2
30   to COOH terminus): Met-Lys-His-His-His-His-His-His-His-Ala-Ser-Asp-Asp-Asp-Asp-Lys (=HEK), followed by amino acids 22 through 401 of the murine OPG polypeptide was placed under control of the lac repressor regulated Ps4 promoter as follows.  pAMG22-His (See Section A) was

- 101 -

digested with NheI and BamHI restriction endonucleases, and the large fragment (the A fragment) isolated from an agarose gel using standard recombinant DNA methodology. Oligonucleotides #1282-91 and #1282-92 were

5    phosphorylated individually and allowed to form an oligo linker duplex using methods previously described (See Section B). The phosphorylated linker duplex formed between oligos #1282-91 and #1282-92 containing NheI and KpnI cohesive ends, the KpnI and BamHI digested and

10   purified PCR product described (see Section D), and the A fragment of vector pAMG22-His digested with NheI and BamHI were ligated using standard recombinant DNA methodology. The ligation was transformed into E. coli host GM120 by electroporation utilizing the

15   manufacturer's protocol. Clones were selected, plasmid DNA isolated and DNA sequencing performed to verify the DNA sequence of the muOPG-HEK[22-401] gene. DNA sequencing revealed a spurious mutation in the natural muOPG sequence that resulted in a single amino acid

20   change of Alanine-45 of muOPG polypeptide to a Threonine.

Expression of recombinant muOPG-HEK[22-401] (A45T) from GM120 cells harboring the recombinant pAMG21 plasmid was determined using methods similar to those

25   described in Section C, except instead of addition of the synthetic autoinducer, IPTG was added to 0.4 mM final to achieve induction.


Oligo #1282-91

30        5'-CTA GCG ACG ACG ACG ACA AAG AAA CTC TGC CTC CAA AAT ACC TGC ATT ACG ATC CGG AAA CTG GTC ATC AGC TGC TGT GTG ATA AAT GTG CTC CGG GTA C-3' (SEQ ID NO:91)

- 102 -

Oligo #1282-92

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT
5    TGT CGT CGT CGT CG-3' (SEQ ID NO:92)


S. Human OPG met-arg-gly-ser-(his)6[22-401]

Eight oligonucleotides (1338-09 to 1338-16
shown below) were designed to produce a 175 base
10    fragment as overlapping, double stranded DNA. The
oligos were annealed, ligated, and the 5' and 3' oligos
were used as PCR primers to produce large quantities of
the 175 base fragment. The final PCR gene products were
digested with restriction endonucleases ClaI and KpnI to
15    yield a fragment which replaces the N-terminal 28 codons
of human OPG. The ClaI and KpnI digested PCR product
was inserted into pAMG21-huOPG [27-401] which had also
been cleaved with ClaI and KpnI. Ligated DNA was
transformed into competent host cells of E. coli strain
20    393. Clones were screened for the ability to produce
the recombinant protein product and to possess the gene
fusion having the correct nucleotide sequence. Protein
expression levels were determined from 50 ml shaker
flask studies. Whole cell lysate and sonic pellet were
25    analyzed for expression of the construct by Coomassie
stained PAGE gels and Western analysis with murine anti-
OPG antibody. Expression of huOPG Met-Arg-Gly-Ser-
(His)6 [22-401] resulting in the formation of large
inclusion bodies and the protein was localized to the
30    insoluble (pellet) fraction.


1338-09

ACA AAC ACA ATC GAT TTG ATA CTA GA (SEQ ID NO:93)

1338-10

TTT GTT TTA ACT AAT TAA AGG AGG AAT AAA ATA TGA GAG GAT CGC ATC AC

(SEQ ID NO:94)


5    1338-11

CAT CAC CAT CAC GAA ACC TTC CCG CCG AAA TAC CTG CAC TAC GAC GAA GA

(SEQ ID NO:95)


1338-12

10   AAC CTC CCA CCA GCT GCT GTG CGA CAA ATG CCC GCC GGG TAC CCA AAC A

(SEQ ID NO:96)


1338-13

TGT TTG GGT ACC CGG CGG GCA TTT GT (SEQ ID NO:97)

15

1338-14

CGC ACA GCA GCT GGT GGG AGG TTT CTT CGT CGT AGT GCA GGT ATT TCG GC

(SEQ ID NO:98)


20   1338-15

GGG AAG GTT TCG TGA TGG TGA TGG TGA TGC GAT CCT CTC ATA TTT TAT T

(SEQ ID NO:99)


1338-16

25   CCT CCT TTA ATT AGT TAA AAC AAA TCT AGT ATC AAA TCG ATT GTG TTT GT

(SEQ ID NO:100)


### T. Human OPG met-lys[22-401] and met(lys)₃[22-401]

To construct the met-lys and met-(lys)3
30   versions of human OPG[22-401], overlapping
oligonucleotides were designed to add the appropriate
number of lysine residues. The two oligos for each
construct were designed to overlap, allowing two rounds
of PCR to produce the final product. The template for

- 104 -

the first PCR reaction was a plasmid DNA preparation
containing the human OPG 22-401 gene. The first PCR
added the lysine residue(s). The second PCR used the
product of the first round and added sequence back to
5    the first restriction site, ClaI.

The final PCR gene products were digested with
restriction endonucleases ClaI and KpnI, which replace
the N-terminal 28 codons of hu OPG, and then ligated
into plasmid pAMG21-hu OPG [27-401] which had been also
10   digested with the two restriction endonucleases.
Ligated DNA was transformed into competent host cells of
E. coli strain 393. Clones were screened for the
ability to produce the recombinant protein product and
to possess the gene fusion having the correct nucleotide
15   sequence. Protein expression levels were determined
from 50 ml shaker flask studies. Whole cell lysate and
sonic pellet were analyzed for expression of the
construct by Coomassie stained PAGE gels and Western
analysis with murine anti-OPG antibody. Neither
20   construct had a detectable level of protein expression
and inclusion bodies were not visible. The DNA
sequences were confirmed by DNA sequencing.

Oligonucleotide primers to prepare Met-Lys huOPG[22-
25   401]:
1338-17
ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT
AAA GGA GGA ATA AAA TG (SEQ ID NO:101)

30   1338-18
CTA ATT AAA GGA GGA ATA AAA TGA AAG AAA CTT TTC CTC CAA
AAT ATC (SEQ ID NO:102)

1338-20

- 105 -

TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID
NO:103)

Oligonucleotide primers to prepare Met-(Lys)$_3$-huOPG[22-
401]:
1338-17
ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT
AAA GGA GGA ATA AAA TG (SEQ ID NO:104)

1338-19
CTA ATT AAA GGA GGA ATA AAA TGA AAA AAA AAG AAA CTT TTC
CTC CAA AAT ATC (SEQ ID NO:105)

1338-20
TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID
NO:106)

### U. Human and Murine OPG [22-401]/Fc Fusions

Four OPG-Fc fusions were constructed where the
Fc region of human IgG1 was fused at the N-terminus of
either human or murine Osteoprotegerin amino acids 22 to
401 (referred to as Fc/OPG [22-401]) or at the C-
terminus (referred to as OPG[22-401]/Fc). Fc fusions
were constructed using the fusion vector pFc-A3
described in Example 7.

All fusion genes were constructed using
standard PCR technology. Template for PCR reactions
were plasmid preparations containing the target genes.
Overlapping oligos were designed to combine the
C-terminal portion of one gene with the N terminal
portion of the other gene. This process allows fusing
the two genes together in the correct reading frame
after the appropriate PCR reactions have been performed.
Initially one "fusion" oligo for each gene was put into

- 106 -

a PCR reaction with a universal primer for the vector carrying the target gene. The complimentary "fusion" oligo was used with a universal primer to PCR the other gene. At the end of this first PCR reaction, two

5    separate products were obtained, with each individual gene having the fusion site present, creating enough overlap to drive the second round of PCR and create the desired fusion. In the second round of PCR, the first two PCR products were combined along with universal

10   primers and via the overlapping regions, the full length fusion DNA sequence was produced.

The final PCR gene products were digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 having been also digested

15   with the two restriction endonucleases. Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein

20   expression levels were determined from 50 ml shaker flask studies. Whole cell lysate, sonic pellet, and supernatant were analyzed for expression of the fusion by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody.

25

Fc/huOPG [22-401]

Expression of the Fc/hu OPG [22-401] fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The cells have very large inclusion

30   bodies, and the majority of the product is in the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

- 107 -

1318-48

CAG CCC GGG TAA AAT GGA AAC GTT TCC TCC AAA ATA TCT TCA
TT (SEQ ID NO:107)


5    1318-49

CGT TTC CAT TTT ACC CGG GCT GAG CGA GAG GCT CTT CTG CGT
GT (SEQ ID NO:108)


Fc/muOPG [22-401]

10          Expression of the fusion peptide was detected
on a Coomassie stained gel and on a Western blot.  The
cells have very large inclusion bodies, and the majority
of the product is in the insoluble (pellet) fraction.
The following primers were used to construct this OPG-Fc
15   fusion:


1318-50

CGC TCA GCC CGG GTA AAA TGG AAA CGT TGC CTC CAA AAT ACC
TGC (SEQ ID NO:109)

20

1318-51

CCA TTT TAC CCG GGC TGA GCG AGA GGC TCT TCT GCG TGT
(SEQ ID NO:110)


25   muOPG [22-401]/Fc

          Expression of the fusion peptide was detected
on a Coomassie stained gel and on a Western blot.  The
amount of recombinant product was less than the OPG
fusion proteins having the Fc region in the N terminal
30   position.  Obvious inclusion bodies were not detected.
Most of the product appeared to be in the insoluble
(pellet) fraction.  The following primers were used to
construct this OPG-Fc fusion:

- 108 -

1318-54

GAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC
(SEQ ID NO:111)

5    1318-55

CAG CTG CAG CTA AGC AGC TTA TTT TCA CGG ATT G
(SEQ ID NO:112)

10    huOPG [22-401]/Fc

              Expression of the fusion peptide was not
detected on a Coomassie stained gel, although a faint
Western positive signal was present.  Obvious inclusion
bodies were not detected.  The following primers were
15    used to prepare this OPG-Fc fusion:

1318-52

AAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC
(SEQ ID NO:113)

20

1318-53

CAG CTG CAG CTA AGC AGC TTA TTT TTA CTG ATT GG
(SEQ ID NO:114)

25    V.  Human OPG met[22-401]-Fc fusion (P25A)

              This construct combines a proline to alanine
amino acid change at position 25 (P25A) with the huOPG
met[22-401]-Fc fusion.  The plasmid was digested with
restriction endonucleases ClaI and KpnI, which removes
30    the N-terminal 28 codons of the gene, and the resulting
small (less than 200 base pair) fragment was gel
purified.  This fragment containing the proline to
alanine change was then ligated into plasmid pAMG21-
huOPG [22-401]-Fc fusion which had been digested with

the two restriction endonucleases.  The ligated DNA was
transformed into competent host cells of E. coli strain
393.  Clones were screened for the ability to produce
the recombinant protein product and to possess the gene
5   fusion having the correct nucleotide sequence.  Protein
expression levels were determined from 50 ml shaker
flask studies.  Whole cell lysate and sonic pellet were
analyzed for expression of the construct by Coomassie
stained PAGE gels and Western analysis with murine anti-
10  OPG antibody.  The expression level of the fusion
peptide was detected on a Coomassie stained PAGE gel and
on a Western blot.  The protein was in the insoluble
(pellet) fraction.  The cells had large inclusion
bodies.

15

W.   Human OPG met[22-401] (P25A)

A DNA sequence coding for an N-terminal
methionine and amino acids 22 through 401 of human OPG
with the proline at position 25 being substituted by
20  alanine under control of the lux $P_R$ promoter in
prokaryotic expression vector pAMG21 was constructed as
follows:  Synthetic oligos # 1289-84 and 1289-85 were
annealed to form an oligo linker duplex with XbaI and
KpnI cohesive ends.  The synthetic linker duplex
25  utilized optimal E. coli codons and encoded an N-
terminal methionine.  The linker also included an SpeI
restriction site which was not present in the original
sequence.  The linker duplex was directionally inserted
between the XbaI and KpnI sites in pAMG21-huOPG-22-401
30  using standard methods.  The ligation mixture was
introduced into E. coli host GM221 by transformation.
Clones were initially screened for production of the
recombinant protein.  Plasmid DNA was isolated from
positive clones and DNA sequencing was performed to

- 110 -

verify the DNA sequence of the HuOPG-Met[22-401](P25A)
gene.  The following oligonucleotides were used to
generate the  XbaI - KpnI linker:

5          Oligo #1289-84

5'-CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC
AAA ATA TCT TCA TTA TGA TGA AGA AAC TAG TCA TCA GCT GCT
GTG TGA TAA ATG TCC GCC GGG TAC -3'  (SEQ ID NO:115)

10

           Oligo #1289-85

5'- CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAC TAG
TTT CTT CAT CAT AAT GAA GAT ATT TTG GAG CAA AAG TTT CCA
TAT GTT ATT CCT CCT T-3'  (SEQ ID NO:116)

15

### X.  Human OPG met[22-401] (P26A) and (P26D)

A DNA sequence coding for an N-terminal
methionine and amino acids 22 through 401 of human OPG
with the proline at position 26 being substituted by
20   alanine under control of the lux PR promoter in
prokaryotic expression vector pAMG21 was constructed as
follows:  Synthetic oligos # 1289-86 and 1289-87 were
annealed to form an oligo linker duplex with XbaI and
SpeI cohesive ends.  The synthetic linker duplex
25   utilized optimal E. coli codons and encoded an N-
terminal methionine.  The linker duplex was
directionally inserted between the XbaI and SpeI sites
in pAMG21-huOPG[22-401](P25A) using standard methods.
The ligation mixture was introduced into E. coli host
30   GM221 by transformation.  Clones were initially screened
for production of the recombinant protein.  Plasmid DNA
was isolated from positive clones and DNA sequencing was
performed to verify the DNA sequence of the huOPG-
met[22-401](P26A) gene.  One of the clones sequenced was

- 111 -

found to have the proline at position 26 substituted by aspartic acid rather than alanine, and this clone was designated huOPG-met[22-401](P26D). The following oligonucleotides were used to generate the XbaI - SpeI linker:

Oligo #1289-86

5' - CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TCC TGC TAA ATA TCT TCA TTA TGA TGA AGA AA - 3'
(SEQ ID NO:117)

Oligo #1289-87

5' - CTA GTT TCT TCA TCA TAA TGA AGA TAT TTA GCA GGA AAA GTT TCC ATA TGT TAT TCC TCC TT - 3'
(SEQ ID NO:118)

Y.  Human OPG met[22-194] (P25A)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 194 of human OPG with the proline at position 25 being substituted by alanine under control of the lux P_R promoter in prokaryotic expression vector pAMG21 was constructed as follows: The plasmids pAMG21-huOPG[27-194] and pAMG21-huOPG[22-401] (P25A) were each digested with KpnI and BamHI endonucleases. The 450 bp fragment was isolated from pAMG21-huOPG[27-194] and the 6.1 kbp fragment was isolated from pAMG21-huOPG[22-401] (P25A). These fragments were ligated together and introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones

- 112 -

and DNA sequencing was performed to verify the DNA
sequence of the huOPG-Met[22-194](P25A) gene.

EXAMPLE 9
Association of OPG Monomers

        CHO cells engineered to overexpress muOPG [22-
401] were used to generate conditioned media for the
analysis of secreted recombinant OPG using rabbit
polyclonal anti-OPG antibodies.  An aliquot of
conditioned media was concentrated 20-fold, then
analysed by reducing and non-reducing SDS-PAGE
(Figure 15).  Under reducing conditions, the protein
migrated as a Mr 50-55 kd polypeptide, as would be
predicted if the mature product was glycosylated at one
or more of its consensus N-linked glycosylation sites.
Suprisingly, when the same samples were analysed by non-
reducing SDS-PAGE, the majority of the protein migrated
as an approximately 100 kd polypeptide, twice the size
of the reduced protein.  In addition, there was a
smaller amount of the Mr 50-55 kd polypeptide.  This
pattern of migration on SDS-PAGE was consistent with the
notion that the OPG product was forming dimers through
oxidation of a free sulfhydryl group(s).
        The predicted mature OPG polypeptide contains
23 cysteine residues, 18 of which are predicted to be
involved in forming intrachain disulfide bridges which
comprise the four cysteine-rich domains (Figure 12A).
The five remaining C-terminal cysteine residues are not
involved in secondary structure which can be predicted
based upon homology with other TNFR family members.
Overall there is a net uneven number of cysteine
residues, and it is formally possible that at least one

- 113 -

residue is free to form an intermolecular disulfide bond
between two OPG monomers.

To help elucidate patterns of OPG kinesis and
monomer association, a pulse-chase labelling study was
5    performed.  CHO cells expressing muOPG [22-401] were
metabolically labelled as described above in serum-free
medium containing $^{35}S$ methionine and cysteine for 30 min.
After this period, the media was removed, and replaced
with complete medium containing unlabelled methionine
10   and cysteine at levels approximately 2,000-fold excess
to the original concentration of radioactive amino
acids.  At 30 min, 1hr, 2 hr, 4 hr, 6 hr and 12 hr post
addition, cultures were harvested by the removal of the
conditioned media, and lysates of the conditioned media
15   and adherent monolayers were prepared.  The culture
media and cell lysates were clarified as described
above, and then immunoprecipitated using anti-OPG
antibodies as described above.  After the
immunoprecipitates were washed, they were released by
20   boiling in non-reducing SDS-PAGE buffer then split into
two equal halves.  To one half, the reducing agent β-
mercaptothanol was added to 5% (v/v) final
concentration, while the other half was maintained in
non-reducing conditions. Both sets of immunoprecipitates
25   were analysed by SDS-PAGE as described above, then
processed for autoradiography and exposed to film.  The
results are shown in Figure 16.  The samples analysed by
reducing SDS-PAGE are depicted in the bottom two panels.
After synthesis, the OPG polypeptide is rapidly
30   processed to a slightly larger polypeptide, which
probably represents modification by N-linked
glycoslyation.  After approximately 1-2 hours, the level
of OPG in the cell decreases dramatically, and
concomitantly appears in the culture supernatant.  This

- 114 -

appears to be the result of the vectoral transport of
OPG from the cell into the media over time, consistent
with the notion that OPG is a naturally secreted
protein.  Analysis of the same immunoprecipitates under
5   nonreducing conditions reveals the relationship between
the formation of OPG dimers and secretion into the
conditioned media (Figure 16, upper panels).  In the
first 30-60 minutes, OPG monomers are processed in the
cell by apparent glycoslylation, followed by dimer
10  formation.  Over time, the bulk of OPG monomers are
driven into dimers, which subsequently disappear from
the cell. Beginning about 60 minutes after synthesis,
OPG dimers appear in the conditioned media, and
accumulate over the duration of the experiment.
15  Following this period, OPG dimers are formed, which are
then secreted into the culture media.  OPG monomers
persist at a low level inside the cell over time, and
small amounts also appear in the media.  This does not
appear to be the result of breakdown of covalent OPG
20  dimers, but rather the production of sub-stoichiometric
amounts of monomers in the cell and subsequent
secretion.

Recombinantly produced OPG from transfected
CHO cells appears to be predominantly a dimer.  To
25  determine if dimerization is a natural process in OPG
synthesis, we analysed the conditioned media of a cell
line found to naturally express OPG.  The CTLL-2 cell
line, a murine cytotoxic T lymphocytic cell line (ATCC
accession no. TIB-214), was found to express OPG mRNA in
30  a screen of tissue and cell line RNA.  The OPG
transcript was found to be the same as the cloned and
sequenced 2.5-3.0 kb RNA identified from kidney and
found to encode a secreted molecule. Western blot
analysis of conditioned media obtained from CTLL-2 cells

- 115 -

shows that  most, if not all, of the OPG protein
secreted is a dimer (Figure 17).  This suggests that OPG
dimerization and secretion is not an artifact of
overexpression in a cell line, but is likely to be the
5    main form of the product as it is produced by expressing
cells.

        Normal and transgenic mouse tissues and serum
were analysed to determine the nature of the OPG
molecule expressed in OPG transgenic mice.  Since the
10   rat OPG cDNA was expressed under the control of a
hepatocyte control element, extracts made from the
parenchyma of control and transgenic mice under non-
reducing conditions were analysed (Figure 18).  In
extract from transgenic, but not control mice, OPG
15   dimers are readily detected, along with
substoichiometric amounts of monomers.  The OPG dimers
and monomers appear identical to the recombinant murine
protein expressed in the genetically engineered CHO
cells.  This strongly suggests that OPG dimers are
20   indeed a natural form of the gene product, and are
likely to be key active components.  Serum samples
obtained from control and transgenic mice were similarly
analysed by western blot analysis.  In control mice, the
majority of OPG protein migrates as a dimer, while small
25   amounts of monomer are also detected.  In addition,
significant amounts of a larger OPG related protein is
detected, which migrates with a relative molecular mass
consistent with the predicted size of a covalently-
linked trimer.  Thus, recombinant OPG is expressed
30   predominantly as a dimeric protein in OPG transgenic
mice, and the dimer form may be the basis for the
osteopetrotic phenotype in OPG mice.  OPG recombinant
protein may also exist in higher molecular weight
"trimeric" forms.

- 116 -

To determine if the five C-terminal cysteine residues of OPG play a role in homodimerization, the murine OPG codons for cytsteine residues 195 (C195), C202, C277, C319, and C400 were changed to serine using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as described above. The muOPG gene was subcloned between the Not I and Xba I sites of the pcDNA 3.1 (+) vector (Invitrogen, San Diego, CA). The resulting plasmid, pcDNA3.1-muOPG, and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliqout of the reaction is then transfected into competent E. coli XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to change the codon for cysteine residue 195 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C195S protein:

1389-19:
    5′ -CAC GCA AAA GTC GGG AAT AGA TGT CAC-3′
(SEQ ID NO:150)

1406-38:
    5′ -GTG ACA TCT ATT CCC GAC TTT TGC GTG-3′
(SEQ ID NO:151)

The following primer pairs were used to change the codon for cysteine residue 202 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C202S protein:

- 117 -


1389-21:

    5' -CAC CCT GTC GGA AGA GGC CTT CTT C-3'
(SEQ ID NO:152)

5


1389-22:

    5' -GAA GAA GGC CTC TTC CGA CAG GGT G-3' (1389-22)
(SEQ ID NO:153)


10          The following primer pairs were used to change
the codon for cysteine residue 277 to serine of the
murine OPG gene, resulting in the production of a muOPG
[22-401] C277S protein:


15    1389-23:

    5' -TGA CCT CTC GGA AAG CAG CGT GCA-3'
(SEQ ID NO:154)


    1389-24:

20    5' -TGC ACG CTG CTT TCC GAG AGG TCA-3'
(SEQ ID NO:155)


          The following primer pairs were used to change
the codon for cysteine residue 319 to serine of the
25    murine OPG gene, resulting in the production of a muOPG
[22-401] C319S protein:


    1389-17:

    5' -CCT CGA AAT CGA GCG AGC AGC TCC-3'
30    (SEQ ID NO:156)


    1389-18:

    5' -CGA TTT CGA GGT CTT TCT CGT TCT C-3'
(SEQ ID NO:157)

- 118 -

The following primer pairs were used to change the codon for cysteine residue 400 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C400S protein:

1406-72:

5'-CCG TGA AAA TAA GCT CGT TAT AAC TAG GAA TGG-3' (SEQ ID NO:158)

1406-75:

5'-CCA TTC CTA GTT ATA ACG AGC TTA TTT TCA CGG-3' (SEQ ID NO:159)

Each resulting muOPG [22-401] plasmid containing the appropriate mutation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in example 11. Conditioned media from each transfectant was analysed by non-reducing SDS-PAGE and western blotting with anti-OPG antibodies.

Mutation of any of the five C-terminal cysteine residues results in the production of predominantly (>90%) monomeric 55 kd OPG molecules. This strongly suggests that the C-terminal cysteine residues together play a role in OPG homodimerization.

C-terminal OPG deletion mutants were constructed to map the region(s) of the OPG C-terminal domain which are important for OPG homodimerization. These OPG mutants were constructed by PCR amplification using primers which introduce premature stop translation

- 119 -

signals in the C-terminal region of murine OPG. The 5'
oligo was designed to the MuOPG start codon (containing
a HindIII restriction site) and the 3' oligonucleotides
(containing a stop codon and XhoI site) were designed to
5  truncate the C-terminal region of muOPG ending at either
threonine residue 200 (CT 200), proline 212 (CT212),
glutamic acid 293 (CT-293), or serine 355 (CT-355).
        The following primers were used to construct
muOPG [22-200]:

10

1091-39:
        5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
AG-3' (SEQ ID NO:160)

15

1391-91:
        5' -CCT CTC TCG AGT CAG GTG ACA TCT ATT CCA CAC TTT
TGC GTG GC-3' (1391-91) (SEQ ID NO:161)

20      The following primers were used to construct muOPG
[22-212]:

1091-39:
        5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA
25  AG-3' (SEQ ID NO:162)
1391-90:
        5' -CCT CTC TCG AGT CAA GGA ACA GCA AAC CTG AAG AAG
GC -3' (SEQ ID NO:163)

30      The following primers were used to construct muOPG
[22-293]:

- 120 -

1091-39:

    5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3' (SEQ ID NO:164)

1391-89:

5

    5'- CCT CTC TCG AGT CAC TCT GTG GTG AGG TTC GAG TGG CC-3' (SEQ ID NO:165)

    The following primers were used to construct muOPG
10    [22-355]:

    1091-39:

    5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA ACA AG-3' (SEQ ID NO:166)

15

    1391-88:

    5' CCT CTC TCG AGT CAG GAT GTT TTC AAG TGC TTG AGG GC-3' (SEQ ID NO:167)

20    Each resulting muOPG-CT plasmid containing the appropriate truncation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in
25 vitro osteoclast forming assay described in example 11. The conditioned medias were also analysed by non-reducing SDS-PAGE and western blotting using anti-OPG antibodies.

    Truncation of the C-terminal region of OPG
30 effects the ability of OPG to form homodimers. CT 355 is predominantly monomeric, although some dimer is formed. CT 293 forms what appears to be equal molar amounts of monomer and dimer, and also high molecular

- 121 -

weight aggregates.  However, CT 212 and CT 200 are monomeric.


## EXAMPLE 10
## Purification of OPG

### A.  Purification of mammalian OPG-Fc Fusion Proteins

5 L of conditioned media from 293 cells expressing an OPG-Fc fusion protein were prepared as follows.  A frozen sample of cells was thawed into 10 ml of 293S media (DMEM-high glucose, 1x L-glutamine, 10% heat inactivated fetal bovine serum (FBS) and 100 ug/ml hygromycin) and fed with fresh media after one day. After three days, cells were split into two T175 flasks at 1:10 and 1:20 dilutions.  Two additional 1:10 splits were done to scale up to 200 T175 flasks.  Cells were at 5 days post-thawing at this point.  Cells were grown to near confluency (about three days) at which time serum-containing media was aspirated, cells were washed one time with 25 ml PBS per flask and 25 ml of SF media (DMEM-high glucose, 1x L-glutamine) was added to each flask.  Cells were maintained at 5% CO2 for three days at which point the media was harvested, centrifuged, and filtered through 0.45m cellulose nitrate filters (Corning).

OPG-Fc fusion proteins were purified using a Protein G Sepharose column (Pharmacia) equilibrated in PBS. The column size varied depending on volume of starting media.  Conditioned media prepared as described above was loaded onto the column, the column washed with PBS, and pure protein eluted using 100mM glycine pH 2.7. Fractions were collected into tubes containing 1M Tris pH 9.2 in order to neutralize as quickly as possible. Protein containing fractions were pooled, concentrated

- 122 -

in either an Amicon Centricon 10 or Centriprep 10 and diafiltered into PBS. The pure protein is stored at - 80°C.

5      Murine [22-401]-Fc, Murine [22-180]-Fc, Murine [22-194]-Fc, human [22-401]-Fc and human [22-201]Fc were purified by this procedure. Murine [22-185]-Fc is purified by this procedure.

B.   Preparation of anti-OPG antibodies

10      Three New Zealand White rabbits (5-8 lbs initial wt) were injected subcutaneously with muOPG[22-401]-Fc fusion protein. Each rabbit was immunized on day 1 with 50 µg of antigen emulsified in an equal volume of Freunds complete adjuvant. Further boosts
15   (Days 14 and 28) were performed by the same procedure with the substitution of Freunds incomplete adjuvant. Antibody titers were monitored by EIA. After the second boost, the antisera revealed high antibody titers and 25ml production bleeds were obtained from each animal.
20   The sera was first passed over an affinity column to which murine OPG-Fc had be immobilized. The anti-OPG antibodies were eluted with Pierce Gentle Elution Buffer containing 1% glacial acetic acid. The eluted protein was then dialyzed into PBS and passed over a Fc column
25   to remove any antibodies specific for the Fc portion of the OPG fusion protein. The run through fractions containing anti-OPG specific antibodies were dialyzed into PBS.

30   C.   Purification of murine OPG[22-401]

Antibody Affinity Chromatography

Affinity purified anti-OPG antibodies were diafiltered into coupling buffer (0.1M sodium carbonate

- 123 -

pH 8.3, 0.5M NaCl), and mixed with CNBr-activated sepharose beads (Pharmacia) for two hours at room temperature. The resin was then washed with coupling buffer extensively before blocking unoccupied sited with
5  1M ethanolamine (pH 8.0) for two hours at room temperature. The resin was then washed with low pH (0.1M sodium acetate pH 4.0, 0.5M NaCl) followed by a high pH wash (0.1M Tris-HCl pH 8.0, 0.5M NaCl). The last washes were repeated three times. The resin was finally
10 equilibrated with PBS before packing into a column. Once packed, the resin was washed with PBS. A blank elution was performed with 0.1M glycine-HCl, pH 2.5), followed by re-equilibration with PBS.

   Concentrated conditioned media from CHO cells
15 expressing muOPG[22-410] was applied to the column at a low flow rate. The column was washed with PBS until UV absorbance measured at 280nm returned to baseline. The protein was eluted from the column first with 0.1M glycine-HCl (pH 2.5), re-equilibrated with PBS, and
20 eluted with a second buffer (0.1M CAPS, pH 10.5), 1M NaCl). The two elution pools were diafiltered separately into PBS and sterile filtered before freezing at -20°C.

Conventional Chromatography

25     CHO cell conditioned media was concentrated 23x in an Amicon spiral wound cartridge (S10Y10) and diafiltered into 20mM tris pH 8.0. The diafiltered media was then applied to a Q-sepharose HP (Pharmacia) column which had been equilibrated with 20mM tris pH 8.0. The
30 column was then washed until absorbence at 280nm reached baseline. Protein was eluted with a 20 column volume gradient of 0-300mM NaCl in tris pH 8.0. OPG protein was detected using a western blot of column fractions.

- 124 -

Fractions containing OPG were pooled and brought to a final concentration of 300mM NaCl, 0.2mM DTT. A NiNTA superose (Qiagen) column was equilibrated with 20mM tris pH 8.0, 300mM NaCl, 0.2mM DTT after which the pooled fractions were applied. The column was washed with equilibration buffer until baseline absorbence was reached. Proteins were eluted from the column with a 0-30mM Imidazole gradient in equilibration buffer. Remaining proteins were washed off the column with 1M Imidazole. Again a western blot was used to detect OPG containing fractions.

Pooled fractions from the NiNTA column were dialyzed into 10mm potassium phosphate pH 7.0, 0.2mM DTT. The dialyzed pool was then applied to a ceramic hydroxyapatite column (Bio-Rad) which had been equilibrated in 10mM phosphate buffer. After column washing, the protein was eluted with a 10-100mM potassium phosphate gradient over 20 column volumes. This was then followed by a 20 column volume gradient of 100-400 mM phosphate.

OPG was detected by coomassie blue staining of SDS-polyacrylamide gels and by western blotting. Fractions were pooled and diafiltered onto PBS and frozen at -80°C. The purified protein runs as a monomer and will remain so after diafiltration into PBS. The monomer is stable when stored frozen or at pH 5 at 4°C. However if stored at 4°C in PBS, dimers and what appears to be trimers and tetramers will form after one week.

D. Purification of human OPG met [22-401] from E. coli

The bacterial cell paste was suspended into 10 mM EDTA to a concentration of 15% (w/v) using a low shear homogenizer at 5°C. The cells were then disrupted

- 125 -

by two homogenizations at 15,000 psi each at 5°C. The resulting homogenate was centrifuged at 5,000 x g for one hour at 5°C. The centrifugal pellet was washed by low shear homogenization into water at the original

5 homogenization volume followed by centrifugation as before. The washed pellet was then solubilized to 15% (w/v) by a solution of (final concentration) 6 M guanidine HCl, 10 mM dithiothreitol, 10 mM TrisHCl, pH 8.5 at ambient temperature for 30 minutes. This

10 solution was diluted 30-fold into 2M urea containing 50 mM CAPS, pH 10.5, 1 mM reduced glutathione and then stirred for 72 hours at 5°C. The OPG was purified from this solution at 25°C by first adjustment to pH 4.5 with acetic acid and then chromatography over a column of

15 SP-HP Sepharose resin equilibrated with 25 mM sodium acetate, pH 4.5. The column elution was carried out with a linear sodium chloride gradient from 50 mM to 550 mM in the same buffer using 20 column volumes at a flow rate of 0.1 column volumes/minute. The peak fractions

20 containing only the desired OPG form were pooled and stored at 5°C or buffer exchanged into phosphate buffered saline, concentrated by ultrafiltration, and then stored at 5°C. This material was analyzed by reverse phase HPLC, SDS-PAGE, limulus amebocyte lysate

25 assay for the presence of endotoxin, and N-terminal sequencing. In addition, techniques such as mass spectrometry, pH/temperature stability, fluoresence, circular dichroism, differential scanning calorimetry, and protease profiling assays may also be used to

30 examine the folded nature of the protein.

- 126 -

## EXAMPLE 11
### Biological Activity of Recombinant OPG

5        Based on histology and histomorphometry, it
appeared that hepatic overexpression of OPG in
transgenic mice markedly decreased the numbers of
osteoclasts leading to a marked increase in bone tissue
(see Example 4).  To gain further insight into potential
mechanism(s) underlying this in vivo effect, various
10 forms of recombinant OPG have been tested in an in vitro
culture model of osteoclast formation (osteoclast
forming assay).  This culture system was originally
devised by Udagawa (Udagawa et al. Endocrinology 125,
1805-1813 (1989), Proc. Natl. Acad. Sci. USA 87, 7260-
15 7264 (1990)) and employs a combination of bone marrow
cells and cells from bone marrow stromal cell lines.  A
description of the modification of this culture system
used for these studies has been previously published
(Lacey et al. Endocrinology 136, 2367-2376 (1995)).  In
20 this method, bone marrow cells, flushed from the femurs
and tibiae of mice, are cultured overnight in culture
media (alpha MEM with 10% heat inactivated fetal bovine
serum) supplemented with 500 U/ml CSF-1 (colony
stimulating factor 1, also called M-CSF), a
25 hematopoietic growth factor specific for cells of the
monocyte/macrophage family lineage.  Following this
incubation, the non-adherent cells are collected,
subjected to gradient purification, and then cocultured
with cells from the bone marrow cell line ST2 (1 x $10^6$
30 non-adherent cells : 1 x $10^5$ ST2 cells/ ml media).  The
media is supplemented with dexamethasone (100 nM) and
the biologically-active metabolite of vitamin D3 known
as 1,25 dihydroxyvitamin D3 (1,25 $(OH)_2$ D3, 10 nM).  To
enhance osteoclast appearance, prostaglandin E2 (250 nM)

- 127 -

is added to some cultures.  The coculture period usually
ranges from 8 - 10 days and the media, with all of the
supplements freshly added, is renewed every 3-4 days.
At various intervals, the cultures are assessed for the
presence of tartrate acid phosphatase (TRAP) using
either a histochemical stain (Sigma Kit # 387A, Sigma,
St. Louis, MO) or TRAP solution assay.  The TRAP
histochemical method allows for the identification of
osteoclasts phenotypically which are multinucleated ($\geq$ 3
nuclei) cells that are also TRAP+.  The solution assay
involves lysing the osteoclast-containing cultures in a
citrate buffer (100 mM, pH 5.0) containing 0.1% Triton
X-100.  Tartrate resistant acid phosphatase activity is
then measured based on the conversion of
p-nitrophenylphosphate (20 nM) to p-nitrophenol in the
presence of 80 mM sodium tartrate which occurs during a
3-5 minute incubation at RT.  The reaction is terminated
by the addition of NaOH to a final concentration of 0.5
M.  The optical density at 405 nm is measured and the
results are plotted.

Previous studies (Udagawa et al. _ibid_) using
the osteoclast forming assay have demonstrated that
these cells express receptors for $^{125}$I-calcitonin
(autoradiography) and can make pits on bone surfaces,
which when combined with TRAP positivity confirm that
the multinucleated cells have an osteoclast phenotype.
Additional evidence in support of the osteoclast
phenotype of the multinucleated cells that arise _in
vitro_ in the osteoclast forming assay are that the cells
express $\alpha$v and $\beta$3 integrins by immunocytochemistry and
calcitonin receptor and TRAP mRNA by in situ
hybridization (ISH).

The huOPG [22-401]-Fc fusion was purified from
CHO cell conditioned media and subsequently utilized in

- 128 -

the osteoclast forming assay. At 100 ng/ml of huOPG [22-401]-Fc, osteoclast formation was virtually 100% inhibited (Figure 19A). The levels of TRAP measured in lysed cultures in microtitre plate wells were also
5   inhibited in the presence of OPG with an $ID_{50}$ of approximately 3 ng/ml (Figure 20). The level of TRAP activity in lysates appeared to correlate with the relative number of osteoclasts seen by TRAP cytochemistry (compare Figures 19A-19G and 20).
10  Purified human IgG1 and TNFbp were also tested in this model and were found to have no inhibitory or stimulatory effects suggesting that the inhibitory effects of the huOPG [22-401]-Fc were due to the OPG portion of the fusion protein. Additional forms of the
15  human and murine molecules have been tested and the cumulative data are summarized in Table 1.


Table 1
20  Effects of various OPG forms on in vitro
osteoclast formation


| OPG Construct | Relative Bioactivity in vitro |
|---|---|
25  |  |  |
| muOPG [22-401]-Fc | +++ |
| muOPG [22-194]-Fc | +++ |
| muOPG [22-185]-Fc | ++ |
| muOPG [22-180]-Fc | - |
30  muOPG [22-401] | +++ |
| muOPG [22-401] C195 | +++ |
| muOPG [22-401] C202 | + |
| muOPG [22-401] C277 | - |
| muOPG [22-401] C319 | + |

- 129 -

| | |
|---|---|
| muOPG [22-401] C400 | + |
| muOPG [22-185] | - |
| muOPG [22-194] | -+ |
| muOPG [22-200] | ++ |
| 5    muOPG [22-212] | - |
| muOPG [22-293] | +++ |
| muOPG [22-355] | +++ |
| | |
| huOPG [22-401]-Fc | +++ |
| 10    huOPG [22-201]-Fc | +++ |
| huOPG [22-401]-Fc P26A | +++ |
| huOPG [22-401]-Fc Y28F | +++ |
| huOPG [22-401] | +++ |
| huOPG [27-401]-Fc | ++ |
| 15    huOPG [29-401]-Fc | ++ |
| huOPG [32-401]-Fc | +/- |

+++,  $ED_{50}$ = 0.4-2 ng/ml

++,   $ED_{50}$ = 2-10 ng/ml

20    +,    $ED_{50}$ = 10-100 ng/ml

-,    $ED_{50}$ > 100 ng/ml


The cumulative data suggest that murine and
human OPG amino acid sequences 22-401 are fully active
25    in vitro, when either fused to the Fc domain, or
unfused.  They inhibit in a dose-dependent manner and
possess half-maximal activities in the 2-10 ng/ml range.
Truncation of the murine C-terminus at threonine residue
180 inactivates the molecule, whereas truncations at
30    cysteine 185 and beyond have full activity.  The
cysteine residue located at position 185 is predicted to
form an SS3 bond in the domain 4 region of OPG. Removal
of this residue in other TNFR-related proteins has
previously been shown to abrogate biological activity

- 130 -

(Yan et al. J. Biol. Chem. 266, 12099-12104 (1994)).
Our finding that muOPG[22-180]-Fc is inactive while
muOPG[22-185]-Fc is active is consistent with these
findings.  This suggests that amino acid residues 22-185
5    define a region for OPG activity.

These findings indicate that like
transgenically-expressed OPG, recombinant OPG protein
also suppressed osteoclast formation as tested in the
osteoclast forming assay.  Time course experiments
10   examining the appearance of TRAP+ cells, β3+ cells,
F480+ cells in cultures continuously exposed to OPG
demonstrate that OPG blocks the appearance TRAP+ and β3+
cells, but not F480+ cells.  In contrast, TRAP+ and β3+
cells begin to appear as early as day 4 following
15   culture establishment in control cultures.  Only F480+
cells can be found in OPG-treated cultures and they
appear to be present at qualitatively the same numbers
as the control cultures.  Thus, the mechanism of OPG
effects in vitro appears to involve a blockade in
20   osteoclast differentiation at a step beyond the
appearance of monocyte-macrophages but before the
appearance of cells expressing either TRAP or β3
integrins.  Collectively these findings indicate that
OPG does not interfere with the general growth and
25   differentiation of monocyte-macrophage precursors from
bone marrow, but rather suggests that OPG specifically
blocks the selective differentiation of osteoclasts from
monocyte-macrophage precursors.

To determine more specifically when in the
30   osteoclast differentiation pathway that OPG was
inhibitory, a variation of the in vitro culture method
was employed.  This variation, described in (Lacey et
al. supra), employs bone marrow macrophages as
osteoclast precursors.  The osteoclast precursors are

- 131 -

derived by taking the nonadherent bone marrow cells after an overnight incubation in CSF-1/M-CSF, and culturing the cells for an additional 4 days with 1,000 - 2,000 U/ml CSF-1. Following 4 days of culture, termed
5  the growth phase, the non-adherent cells are removed. The adherent cells, which are bone marrow macrophages, can then be exposed for up to 2 days to various treatments in the presence of 1,000 - 2,000 U/ml CSF-1. This 2 day period is called the intermediate
10  differentiation period. Thereafter, the cell layers are again rinsed and then ST-2 cells (1 X $10^5$ cell/ml), dexamethasone (100 nM) and 1,25 (OH)2 D3 (10 nM) are added for the last 8 days for what is termed the terminal differentiation period. Test agents can be
15  added during this terminal period as well. Acquisition of phenotypic markers of osteoclast differentiation are acquired during this terminal period (Lacey et al. ibid).

huOPG [22-401]-Fc (100 ng/ml) was tested for
20  its effects on osteoclast formation in this model by adding it during either the intermediate, terminal or, alternatively, both differentiation periods. Both TRAP cytochemistry and solution assays were performed. The results of the solution assay are shown in Figure 21.
25  HuOPG [22-401]-Fc inhibited the appearance of TRAP activity when added to both the intermediate and terminal or only the terminal differentiation phases. When added to the intermediate phase and then removed from the cultures by rinsing, huOPG [22-401]-Fc did not
30  block the appearance of TRAP activity in culture lysates. The cytochemistry results parallel the solution assay data. Collectively, these observations indicate that huOPG [22-401]-Fc only needs to be present during the terminal differentiation period for it to

- 132 -

exert its all of its suppressive effects on osteoclast formation.

B. In vivo IL1-α and IL1-β challenge experiments

IL1 increases bone resorption both
5   systemically and locally when injected subcutaneously over the calvaria of mice (Boyce et al., Endocrinology 125, 1142-1150 (1989)). The systemic effects can be assessed by the degree of hypercalcemia and the local effects histologically by assessing the relative
10  magnitude of the osteoclast-mediated response. The aim of these experiments was to determine if recombinant muOPG [22-401]-Fc could modify the local and/or systemic actions of IL1 when injected subcutaneously over the same region of the calvaria as IL1.
15

IL-1 β experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group: IL1 treated animals (mice
20  received 1 injection/day of 2.5 ug of IL1-β); Low dose muOPG [22-401]-Fc treated animals (mice received 3 injections/day of 1 μg of muOPG [22-401]-Fc); Low dose muOPG [22-401]-Fc and IL1-β; High dose muOPG [22-401]-Fc treated animals (mice receive 3 injections/day of 10
25  μg muOPG [22-401]-Fc); High dose muOPG [22-401]-Fc and IL1-β. All mice received the same total number of injections of either active factor or vehicle (0.1% bovine serum albumin in phosphate buffered saline). All groups are sacrificed on the day after the last
30  injection. The weights and blood ionized calcium levels are measured before the first injections, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed.

- 133 -

After sacrifice the calvaria were removed and processed for paraffin sectioning.

### IL1-α experiment

5          Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group; IL1 alpha treated animals (mice received 1 injection/day of 5 ug of IL1-alpha); Low dose muOPG [22-401]-Fc treated animals (mice received 1
10    injection/day of 10 μg of muOPG [22-401]-Fc; Low dose muopg [22-401]-Fc and IL1-alpha, (dosing as above); High dose muopg [22-401]-Fc treated animals (mice received 3 injections/day of 10 μg muOPG [22-401]-Fc; High dose muOPG [22-401]-Fc and IL1-α. All mice received the same
15    number of injections/day of either active factor or vehicle. All groups were sacrificed on the day after the last injection. The blood ionized calcium levels were measured before the first injection, four hours after the second injection and 24 hours after the third
20    IL1 injection, just before the animals were sacrificed. The animal weights were measured before the first injection, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed. After sacrifice the calvaria
25    were removed and processed for paraffin sectioning.

### Histological methods

          Calvarial bone samples were fixed in zinc
30    formalin, decalcified in formic acid, dehydrated through ethanol and mounted in paraffin. Sections (5μm thick) were cut through the calvaria adjacent to the lambdoid suture and stained with either hematoxylin and eosin or reacted for tartrate resistant acid phosphatase activity

- 134 -

(Sigma Kit# 387A) and counterstained with hematoxylin.
Bone resorption was assessed in the IL1-α treated mice
by histomorphometric methods using the Osteomeasure
(Osteometrics, Atlanta, GA) by tracing histologic
5    features onto a digitizor platen using a microscope-
mounted camera lucida attachment.  Osteoclast numbers,
osteoclast lined surfaces, and eroded surfaces were
determined in the marrow spaces of the calvarial bone.
The injected and non-injected sides of the calvaria were
10   measured separately.


Results

          IL1-α and IL1-β produced hypercalcemia at the
doses used, particularly on the second day, presumably
15   by the induction of increased bone resorption
systemically.  The hypercalcemic response was blocked by
muOPG [22-401]-Fc in the IL1-beta treated mice and
significantly diminished in mice treated with IL1-alpha,
an effect most apparent on day 2 (Figure 22A-22B).
20            Histologic analysis of the calvariae of mice
treated with IL1-alpha and beta shows that IL1
treatments alone produce a marked increase in the
indices of bone resorption including: osteoclast number,
osteoclast lined surface, and eroded surface (surfaces
25   showing deep scalloping due to osteoclastic action
(Figure 23B, Table 2).  In response to IL1-α or IL1-β,
the increases in bone resorption were similar on the
injected and non-injected sides of the calvaria.  Muopg
[22-401]-Fc injections reduced bone resorption in both
30   IL1-alpha and beta treated mice and in mice receiving
vehicle alone but this reduction was seen only on the
muopg [22-401]-Fc injected sides of the calvariae.
          The most likely explanation for these
observations is that muOPG [22-401]-Fc inhibited bone

- 135 -

resorption, a conclusion supported by the reduction of both the total osteoclast number and the percentage of available bone surface undergoing bone resorption, in the region of the calvaria adjacent to the muOPG [22-

5    401]-Fc injection sites.  The actions of muOPG [22-401]-Fc appeared to be most marked locally by histology, but the fact that muOPG [22-401]-Fc also blunted IL1-induced hypercalcemia suggests that muOPG [22-401]-Fc has more subtle effects on bone resorption systemically.

Table 2. Effects of OPG on variables of bone resorption in IL-1 injected mice.

| | Osteoclast Surface % Bone Surface (mean ± S.D) | | Eroded Surface %Bone Surface (mean ± S.D) | | Osteoclast Number/mm² Tissue Area (mean ± S.D) | |
|---|---|---|---|---|---|---|
| | Non-injected side | Injected side | Non-injected side | Injected side | Non-injected side | Injected side |
| **Experiment 1** | | | | | | |
| Control | 12.36 ± 3.44 | 9.54 ± 2.46 | 8.07 ± 3.90 | 9.75 ± 3.16 | 32.51 ± 11.09 | 23.50 ± 10.83 |
| IL1-β (2.5µg/d) | 17.18 ± 1.30 | 16.40 ± 2.16 | 40.66 ± 4.28 | 37.53 ± 10.28 | 71.80 ± 18.76 | 60.89 ± 5.16 |
| OPG (40µg/d) | 10.12 ± 3.71 | 5.04 ± 1.66 | 9.73 ± 4.33 | 4.19 ± 3.61 | 32.73 ± 11.09 | 15.24 ± 7.54 |
| OPG+IL1-β | 18.61 ± 2.46 | # 13.26 ± 2.50 | 44.87 ± 8.63 | # 25.94 ± 6.82 | 69.42 ± 36.29 | # 47.13 ± 24.26 |
| **Experiment 2** | | | | | | |
| Control | 11.56 ± 4.22 | 11.95 ± 2.97 | 12.67 ± 5.04 | 10.03 ± 5.13 | 51.72 ± 23.93 | 56.03 ± 30.70 |
| IL1-α (5µg/d) | 28.81 ± 4.84 | 23.46 ± 5.76 | 37.51 ± 5.16 | 41.10 ± 12.53 | 113.60 ± 18.04 | 102.70 ± 32.09 |
| OPG (40µg/d) | 14.40 ± 1.00 | # 4.26 ± 2.54 | 11.55 ± 4.14 | # 4.29 ± 3.16 | 72.28 ± 14.11 | # 22.65 ± 16.68 |
| OPG+IL1-α | 29.58 ± 8.80 | # 17.83 ± 3.34 | 33.66 ± 9.21 | # 24.38 ± 8.88 | 146.10 ± 42.37 | # 66.56 ± 15.62 |

# Different to non-injected side $p < 0.05$ (by paired t test)

- 137 -

### C. Systemic Effects of muOPG [22-401]-Fc in Growing Mice

Male BDF1 mice aged 3-4 weeks, weight range
9.2- 15.7g were divided into groups of ten mice per
group.  These mice were injected subcutaneously with
5  saline or muOPG [22-401]-Fc 2.5mg/kg *bid* for 14 days
(5mg/kg/day).  The mice were radiographed before
treatment, at day 7 and on day 14.  The mice were
sacrificed 24 hours after the final injection.  The
right femur was removed, fixed in zinc formalin,
10  decalcified in formic acid and embedded in paraffin.
Sections were cut through the mid region of the distal
femoral metaphysis and the femoral shaft.  Bone density,
by histomorphometry, was determined in six adjacent
regions extending from the metaphyseal limit of the
15  growth plate, through the primary and secondary
spongiosa and into the femoral diaphysis (shaft).  Each
region was 0.5 X 0.5 mm$^2$.

### Radiographic changes

20                After seven days of treatment there was
evidence of a zone of increased bone density in the
spongiosa associated with the growth plates in the OPG
treated mice relative to that seen in the controls.  The
effects were particularly striking in the distal femoral
25  and the proximal tibial metaphases (Figure 24A-24B).
However bands of increased density were also apparent in
the vertebral bodies, the iliac crest and the distal
tibia.  At 14 days, the regions of opacity had extended
further into the femoral and tibial shafts though the
30  intensity of the radio-opacity was diminished.
Additionally, there were no differences in the length of
the femurs at the completion of the experiment or in the
change in length over the duration of the experiment
implying that OPG does not alter bone growth.

- 138 -

Histological Changes

The distal femoral metaphysis showed increased bone density in a regions 1.1 to 2.65 mm in distance from the growth plate (Figures 25 and 26A-26B). This is a region where bone is rapidly removed by osteoclast-mediated bone resorption in mice. In these rapidly growing young mice, the increase in bone in this region observed with OPG treatment is consistent with an inhibition of bone resorption.

D.    Effects of Osteoprotegerin on Bone Loss Induced by Ovariectomy in the Rat

Twelve week old female Fisher rats were ovariectomized (OVX) or sham operated and dual xray absorptiometry (DEXA) measurements made of the bone density in the distal femoral metaphysis.    After 3 days recovery period, the animals received daily injections for 14 days as follows: Ten  sham operated animals received vehicle (phosphate buffered saline); Ten OVX animals received vehicle (phosphate buffered saline); Six OVX animals received OPG-Fc  5mg/kg SC;   Six OVX animals received pamidronate (PAM) 5mg/kg SC; Six OVX animals received estrogen (ESTR) 40ug/kg SC.   After 7 and 14 days treatment the animals had bone density measured by DEXA.  Two days after the last injection the animals were killed and the right tibia and femur removed for histological evaluation.

The DEXA measurements of bone density showed a trend to reduction in the bone density following ovariectomy that was blocked by OPG-Fc.   Its effects were similar to the known antiresorptive agents estrogen and pamidronate. (Figure 27).  The histomorphometric

- 139 -

analysis confirmed these observations with OPG-Fc treatment producing a bone density that was significantly higher in OVX rats than that seen in untreated OVX rats (Figure 28). These results confirm the activity of OPG in the bone loss associated with withdrawal of endogenous estrogen following ovariectomy.


### In vivo Summary

The in vivo actions of recombinant OPG parallel the changes seen in OPG transgenic mice. The reduction in osteoclast number seen in the OPG transgenic is reproduced by injecting recombinant OPG locally over the calvaria in both normal mice and in mice treated with IL1-$\alpha$ or IL1-$\beta$. The OPG transgenic mice develop an osteopetrotic phenotype with progressive filling of the marrow cavity with bone and unremodelled cartilage extending from the growth plates from day 1 onward after birth. In normal three week old (growing) mice, OPG treatments also led to retention of bone and unremodelled cartilage in regions of endochondral bone formation, an effect observed radiographically and confirmed histologically. Thus, recombinant OPG produces phenotypic changes in normal animals similar to those seen in the transgenic animals and the changes are consistent with OPG-induced inhibition of bone resorption. Based on in vitro assays of osteoclast formation, a significant portion of this inhibition is due to impaired osteoclast formation. Consistent with this hypothesis, OPG blocks ovariectomy-induced osteoporosis in rat. Bone loss in this model is known to be mediated by activated osteoclasts, suggesting a role for OPG in treatment of primary osteoporosis.

- 140 -

## EXAMPLE 12
### Pegylation Derivatives of OPG

5   Preparation of N-terminal PEG-OPG conjugates by
    reductive alkylation

HuOPG met [22-194] P25A was buffer exchanged into
25-50 mM NaOAc, pH 4.5-4.8 and concentrated to 2-5
mg/ml.  This solution was used to conduct OPG reductive
10  alkylation with monofunctional PEG aldehydes at 5-7 °C.
PEG monofunctional aldehydes, linear or branched, MW=1
to 57 kDa (available from Shearwater Polymers) were
added to the OPG solution as solids in amounts
constituting 2-4 moles of PEG aldehyde per mole of OPG.
15  After dissolution of polymer into the protein solution,
sodium cyanoborohydride was added to give a final
concentration of 15 to 20 mM in the reaction mixture
from 1-1.6 M freshly prepared stock solution in cold DI
water. The progress of the reaction and the extent of
20  OPG PEGylation was monitored by size exclusion HPLC on a
G3000SW$_{XL}$ column (Toso Haas) eluting with 100 mM NaPO$_4$,
0.5 M NaCl, 10% ethanol, pH 6.9.  Typically the reaction
was allowed to proceed for 16-18 hours, after which the
reaction mixture was diluted 6-8 times and the pH
25  lowered to 3.5-4.  The reaction mixture was fractionated
by ion exchange chromatography (HP SP HiLoad 16/10,
Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear
gradient to 0.75M NaCl over 25 column volumes at a flow
rate of 30 cm/h.  Fractions of mono-, di- or poly-
30  PEGylated OPG were pooled and characterized by SEC HPLC
and SDS-PAGE.  By N-terminal sequencing, it was
determined that the monoPEG-OPG conjugate, the major
reaction product in most cases, was 98% N-terminally
PEG-modified OPG.

- 141 -

This procedure was generally used to prepare the following N-terminal PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A: 5 kD monoPEG, 10 kD mono branched PEG, 12 kD monoPEG, 20 kD monoPEG, 20 kD mono branched PEG, 25 kD monoPEG, 31 kD monoPEG, 57 kD monoPEG, 12 kD diPEG, 25 kD diPEG, 31 kD diPEG, 57 kD diPEG, 25 kD triPEG.

## Preparation of PEG-OPG conjugates by acylation

HuOPG met [22-194] P25A was buffer exchanged into 50 mM BICINE buffer, pH 8 and concentrated to 2-3 mg/ml. This solution was used to conduct OPG acylation with monofunctional PEG N-hydroxysuccinimidyl esters at room temperature. PEG N-hydroxysuccinimidyl esters, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 4-8 moles of PEG N-hydroxysuccinimidyl ester per mole of OPG. The progress of the reaction and the extent of OPG PEGylation was monitored by size exclusion HPLC on a G3000SW$_{XL}$ column (Toso Haas) eluting with 100 mM NaPO4, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 1 hour, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE.

This procedure was generally used to prepare the following PEG-OPG conjugates: 5 kD polyPEG, 20 kD polyPEG, 40 kD poly branched PEG, 50 kD poly PEG.

- 142 -

Preparation of dimeric PEG-OPG

5      HuOPG met [22-194] P25A is prepared for thiolation at 1-3 mg/ml in a phosphate buffer at near neutral pH. S-acetyl mecaptosuccinic anhydride (AMSA) is added in a 3-7 fold molar excess while maintaining pH at 7.0 and the rxn stirred at 4°C for 2 hrs. The monothiolated-OPG is separated from unmodified and
10     polythiolated OPG by ion exchange chromatography and the protected thiol deprotected by treatment with hydroxylamine. After deprotection, the hydroxylamine is removed by gel filtration and the resultant monothiolated-OPG is subjected to a variety of thiol
15     specific crosslinking chemistries. To generate a disulfide bonded dimer, the thiolated OPG at >1mg/ml is allowed to undergo air oxidation by dialysis in slightly basic phosphate buffer. The covalent thioether OPG dimer was prepared by reacting the bis-maleimide
20     crosslinker, N,N-bis(3-maleimido propianyl)-2-hydroxy 1,3 propane with the thiolated OPG at >1mg/ml at a 0.6x molar ratio of crosslinker:OPG in phosphate buffer at pH 6.5. Similarly, the PEG dumbbells are produced by reaction of substoichiometric amounts of bis-maleimide
25     PEG crosslinkers with thiolated OPG at >1mg/ml in phosphate buffer at pH 6.5. Any of the above dimeric conjugates may be further purified using either ion exchange or size exclusion chromatographies.
       Dimeric PEG-OPG conjugates (where OPG is HuOPG met
30     [22-194] P25A prepared using the above procedures include disulfide-bonded OPG dimer, covalent thioether OPG dimer with an aliphatic amine type crosslinker, 3.4 kD and 8kD PEG dumbbells and monobells.

- 143 -

PEG-OPG conjugates were tested for activity in vitro using the osteoclast maturation assay described in Example 11A and for activity in vivo by measuring increased bone density after injection into mice as

5   described in Example 11C.  The in vivo activity is shown below in Table 3.


Table 3
In vivo biological activity of Pegylated OPG

10

| OPG Construct | Increase in Tibial Bone Density |
|---|---|
| muOPG met [22-194] | - |
| muOPG met [22-194] 5k PEG | + |
| muOPG met [22-194] 20k PEG | + |
| | |
| huOPG met [22-194]    P25A | - |
| huOPG met [22-194] P25A 5k PEG | + |
| huOPG met [22-194] P25A 20k PEG | + |
| huOPG met [22-194] P25A 31k PEG | + |
| huOPG met [22-194] P25A 57k PEG | + |
| huOPG met [22-194] P25A 12k PEG | + |
| huOPG met [22-194] P25A 20k Branched PEG | + |
| huOPG met [22-194] P25A 8k PEG dimer | + |
| huOPG met [22-194] P25A disulfide crosslink | + |

- 144 -

*           *           *

While the invention has been described in what is considered to be its preferred embodiments, it is not
5   to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such
10  modifications and equivalents.

- 145 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:Amgen Inc.

    (ii) TITLE OF INVENTION: OSTEOPROTEGERIN

    (iii) NUMBER OF SEQUENCES: 168

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Amgen Inc.
        (B) STREET: 1840 Dehavilland Drive
        (C) CITY: Thousand Oaks
        (D) STATE: California
        (E) COUNTRY: United States
        (F) ZIP: 91320

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Winter, Robert B.
        (C) REFERENCE/DOCKET NUMBER: A-378-CIP2

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 146 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAAGGAAGGA AAAAAGCGGC CGCTACANNN NNNNNT                                  36

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACCCACG CGTCCG                                                        16

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTGCGCAG GC                                                           12

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 147 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTAAAACGA CGGCCAGT                                                    18

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 18 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGGAAACAG CTATGACC                                                    18

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 20 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAATTAACCC TCACTAAAGG                                                  20

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 23 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

- 148 -

GCATTATGAC CCAGAAACCG GAC

(2) INFORMATION FOR SEQ ID NO:8:                                             23

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGTAGCGCC CTTCCTCACA TTC

(2) INFORMATION FOR SEQ ID NO:9:                                             23

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GACTAGTCCC ACAATGAACA AGTGGCTGTG

(2) INFORMATION FOR SEQ ID NO:10:                                            30

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAAGAATGC GGCCGCTAAA CTATGAAACA GCCCAGTGAC CATTC                            45

(2) INFORMATION FOR SEQ ID NO:11:

- 149 -

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 21 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
    5       (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA


    10  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

        GCCTCTAGAA AGAGCTGGGA C                                          21

        (2) INFORMATION FOR SEQ ID NO:12:
    15
            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 21 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
    20          (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: cDNA


    25  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

        CGCCGTGTTC CATTTATGAG C                                          21

        (2) INFORMATION FOR SEQ ID NO:13:
    30
            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 24 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
    35          (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: cDNA


    40  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

        ATCAAAGGCA GGGCATACTT CCTG                                       24

        (2) INFORMATION FOR SEQ ID NO:14:
    45
            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 24 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
    50          (D) TOPOLOGY: linear

- 150 -

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTGCACTCC TGTTTCACGG TCTG

24

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 24 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAAGACACCT TGAAGGGCCT GATG

24

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 24 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAACTTTTAC AGAAGAGCAT CAGC

24

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 33 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

5        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCGCGGCCG CATGAACAAG TGGCTGTGCT GCG

33

(2) INFORMATION FOR SEQ ID NO:18:

10          (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 31 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

15

       (ii) MOLECULE TYPE: cDNA

20

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTCTAGAG AAACAGCCCA GTGACCATTC C

31

25 (2) INFORMATION FOR SEQ ID NO:19:

         (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 24 base pairs
           (B) TYPE: nucleic acid
30            (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: cDNA

35

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

40 GTGAAGCTGT GCAAGAACCT GATG

24

(2) INFORMATION FOR SEQ ID NO:20:

         (i) SEQUENCE CHARACTERISTICS:
45            (A) LENGTH: 24 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

50        (ii) MOLECULE TYPE: cDNA

- 152 -


(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATCAAAGGCA GGGCATACTT CCTG                                          24

(2) INFORMATION FOR SEQ ID NO:21:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 24 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAGATCCTGA AGCTGCTCAG TTTG                                          24

(2) INFORMATION FOR SEQ ID NO:22:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 33 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGCGCGGCCG CGGGGACCAC AATGAACAAG TTG                                33

(2) INFORMATION FOR SEQ ID NO:23:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 33 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

- 153 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

5      AGCTCTAGAA TTGTGAGGAA ACAGCTCAAT GGC                                    33

(2) INFORMATION FOR SEQ ID NO:24:

            (i) SEQUENCE CHARACTERISTICS:
10              (A) LENGTH: 39 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

15      (ii) MOLECULE TYPE: cDNA


20      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

        ATAGCGGCCG CTGAGCCCAA ATCTTGTGAC AAAACTCAC                            39

        (2) INFORMATION FOR SEQ ID NO:25:

25          (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 45 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
30              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA


35  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

    TCTAGAGTCG ACTTATCATT TACCCGGAGA CAGGGAGAGG CTCTT                         45

    (2) INFORMATION FOR SEQ ID NO:26:

40          (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 38 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
45              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA


50

- 154 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG                                    38

5    (2) INFORMATION FOR SEQ ID NO:27:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 43 base pairs
             (B) TYPE: nucleic acid
10           (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

15

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

20   CCTCTGCGGC CGCTAAGCAG CTTATTTTCA CGGATTGAAC CTG                          43

     (2) INFORMATION FOR SEQ ID NO:28:

         (i) SEQUENCE CHARACTERISTICS:
25           (A) LENGTH: 38 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

30    (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

35   CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG                                38

     (2) INFORMATION FOR SEQ ID NO:29:

40       (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 24 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear
45

      (ii) MOLECULE TYPE: cDNA

50

- 155 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCCGTAAGAA ACAGCCCAGT GACC

24

5    (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
10            (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

20   CCTCTGCGGC CGCTGTTGCA TTTCCTTTCT G

31

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
25            (A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30   (ii) MOLECULE TYPE: protein

35   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His
1               5                   10                  15

Gln Leu Leu
40

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
45            (A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50   (ii) MOLECULE TYPE: cDNA

- 156 -

```
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:
5
TCCCTTGCCC TGACCACTCT T
                                                          21

    (2) INFORMATION FOR SEQ ID NO:33:

10        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 34 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear
15
         (ii) MOLECULE TYPE: cDNA


20
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCTCTGCGGC CGCACACACG TTGTCATGTG TTGC
                                                          34
25   (2) INFORMATION FOR SEQ ID NO:34:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 21 base pairs
              (B) TYPE: nucleic acid
30            (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: cDNA
35


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:
40 TCCCTTGCCC TGACCACTCT T
                                                          21
    (2) INFORMATION FOR SEQ ID NO:35:

          (i) SEQUENCE CHARACTERISTICS:
45            (A) LENGTH: 34 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

50       (ii) MOLECULE TYPE: cDNA
```

- 157 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCTCTGCGGC CGCCTTTTGC GTGGCTTCTC TGTT   34

(2) INFORMATION FOR SEQ ID NO:36:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 37 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG   37

(2) INFORMATION FOR SEQ ID NO:37:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 38 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCTCTGCGGC CGCTAAGCAG CTTATTTTTA CTGAATGG   38

(2) INFORMATION FOR SEQ ID NO:38:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 37 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 158 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG   37

(2) INFORMATION FOR SEQ ID NO:39:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCTCTGCGGC CGCCAGGGTA ACATCTATTC CAC   33

(2) INFORMATION FOR SEQ ID NO:40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CCGAAGCTTC CACCATGAAC AAGTGGCTGT GCTGC   35

(2) INFORMATION FOR SEQ ID NO:41:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 40 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCTCTGTCGA CTATTATAAG CAGCTTATTT TCACGGATTG   40

(2) INFORMATION FOR SEQ ID NO:42:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 21 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TCCCTTGCCC TGACCACTCT T   21

(2) INFORMATION FOR SEQ ID NO:43:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 35 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCTCTGTCGA CTTAACACAC GTTGTCATGT GTTGC   35

(2) INFORMATION FOR SEQ ID NO:44:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 21 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 160 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TCCCTTGCCC TGACCACTCT T   21

(2) INFORMATION FOR SEQ ID NO:45:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 35 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCTCTGTCGA CTTACTTTTG CGTGGCTTCT CTGTT   35

(2) INFORMATION FOR SEQ ID NO:46:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 1537 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTGAAGAGCG TGAAGAGCGG TTCCTCCTTT CAGCAAAAAA CCCCTCAAGA CCCGTTTAGA   60

GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAACT CAGCTTCCTT   120

TCGGGCTTTC TTCTTCTTCT TCTTCTTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC   180

CTGCAGGTCG ACACTAGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCTCCTT   240

TAATTAGTTA AAACAAATCT AGAATCAAAT CGATTAATCG ACTATAACAA ACCATTTTCT   300

TGCGTAAACC TGTACGATCC TACAGGTACT TATGTTAAAC AATTGTATTT CAAGCGATAT   360

AATAGTGTGA CAAAAATCCA ATTTATTAGA ATCAAATGTC AATCTATTAC CGTTTTAATG   420

ATATATAACA CGCAAAACTT GCGACAAACA ATAGGTAAGG ATAAAGAGAT GGGTATGAAA   480

```
GACATAAATG CCGACGACAC TTACAGAATA ATTAATAAAA TTAAAGCCTG TAGAAGCAAT    540

AATGATATTA ATCAATGCTT ATCTGATATG ACTAAAATGG TACATTGTGA ATATTATTTA    600

CTCGCGATCA TTTATCCTCA TTCTATGGTT AAATCTGATA TTTCAATTCT GGATAATTAC    660

CCTAAAAAAT GGAGGCAATA TTATGATGAC GCTAATTTAA TAAAATATGA TCCTATAGTA    720

GATTATTCTA ACTCCAATCA TTCACCGATT AATTGGAATA TATTTGAAAA CAATGCTGTA    780

AATAAAAAAT CTCCAAATGT AATTAAAGAA GCGAAATCAT CAGGTCTTAT CACTGGGTTT    840

AGTTTCCCTA TTCATACTGC TAATAATGGC TTCGGAATGC TTAGTTTTGC ACATTCAGAG    900

AAAGACAACT ATATAGATAG TTTATTTTTA CATGCGTGTA TGAACATACC ATTAATTGTT    960

CCTTCTCTAG TTGATAATTA TCGAAAAATA AATATAGCAA ATAATAAATC AAACAACGAT   1020

TTAACCAAAA GAGAAAAAGA ATGTTTAGCG TGGGCATGCG AAGGAAAAAG CTCTTGGGAT   1080

ATTTCAAAAA TATTAGGCTG TAGTAAGCGC ACGGTCACTT TCCATTTAAC CAATGCGCAA   1140

ATGAAACTCA ATACAACAAA CCGCTGCCAA AGTATTTCTA AAGCAATTTT AACAGGAGCA   1200

ATTGATTGCC CATACTTTAA AAGTTAAGTA CGACGTCCAT ATTTGAATGT ATTTAGAAAA   1260

ATAAACAAAA GAGTTTGTAG AAACGCAAAA AGGCCATCCG TCAGGATGGC CTTCTGCTTA   1320

ATTTGATGCC TGGCAGTTTA TGGCGGGCGT CCTGCCCGCC ACCCTCCGGG CCGTTGCTTC   1380

GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCCTACTCA GGAGAGCGTT CACCGACAAA   1440

CAACAGATAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT TTCGTTTTAT TTGATGCCTG   1500

GCAGTTCCCT ACTCTCGCAT GGGGAGACCA TGCATAC                           1537
```

(2) INFORMATION FOR SEQ ID NO:47:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 48 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 162 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA   48

(2) INFORMATION FOR SEQ ID NO:48:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 55 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGGAATTC GGTAC   55

(2) INFORMATION FOR SEQ ID NO:49:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 49 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CGAATTCCAA CGCGTTAACC ATATGTTATT CCTCCTTCTA GAATCAAAT   49

(2) INFORMATION FOR SEQ ID NO:50:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 1546 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 163 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

```
      GCGTAACGTA TGCATGGTCT CCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA        60
  5   CGAAAGGCTC AGTCGAAAGA CTGGGCCTTT CGTTTTATCT GTTGTTTGTC GGTGAACGCT       120
      CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG TTGCGAAGCA ACGGCCCGGA       180
 10   GGGTGGCGGG CAGGACGCCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGCCATC       240
      CTGACGGATG GCCTTTTTGC GTTTCTACAA ACTCTTTTGT TTATTTTTCT AAATACATTC       300
      AAATATGGAC GTCGTACTTA ACTTTTAAAG TATGGGCAAT CAATTGCTCC TGTTAAAATT       360
 15   GCTTTAGAAA TACTTTGGCA GCGGTTTGTT GTATTGAGTT TCATTTGCGC ATTGGTTAAA       420
      TGGAAAGTGA CCGTGCGCTT ACTACAGCCT AATATTTTTG AAATATCCCA GAGCTTTTT        480
      CCTTCGCATG CCCACGCTAA ACATTCTTTT TCTCTTTTGG TTAAATCGTT GTTTGATTTA       540
 20   TTATTTGCTA TATTTATTTT TCGATAATTA TCAACTAGAG AAGGAACAAT TAATGGTATG       600
      TTCATACACG CATGTAAAAA TAAACTATCT ATATAGTTGT CTTTCTCTGA ATGTGCAAAA       660
      CTAAGCATTC CGAAGCCATT ATTAGCAGTA TGAATAGGGA AACTAAACCC AGTGATAAGA       720
 25   CCTGATGATT TCGCTTCTTT AATTACATTT GGAGATTTTT TATTTACAGC ATTGTTTTCA       780
      AATATATTCC AATTAATCGG TGAATGATTG GAGTTAGAAT AATCTACTAT AGGATCATAT       840
 30   TTTATTAAAT TAGCGTCATC ATAATATTGC CTCCATTTTT TAGGGTAATT ATCCAGAATT       900
      GAAATATCAG ATTTAACCAT AGAATGAGGA TAAATGATCG CGAGTAAATA ATATTCACAA       960
 35   TGTACCATTT TAGTCATATC AGATAAGCAT TGATTAATAT CATTATTGCT TCTACAGGCT      1020
      TTAATTTTAT TAATTATTCT GTAAGTGTCG TCGGCATTTA TGTCTTTCAT ACCCATCTCT      1080
 40   TTATCCTTAC CTATTGTTTG TCGCAAGTTT TGCGTGTTAT ATATCATTAA AACGGTAATA      1140
      GATTGACATT TGATTCTAAT AAATTGGATT TTTGTCACAC TATTATATCG CTTGAAATAC      1200
      AATTGTTTAA CATAAGTACC TGTAGGATCG TACAGGTTTA CGCAAGAAAA TGGTTTGTTA      1260
 45   TAGTCGATTA ATCGATTTGA TTCTAGATTT GTTTTAACTA ATTAAAGGAG GAATAACATA      1320
      TGGTTAACGC GTTGGAATTC GAGCTCACTA GTGTCGACCT GCAGGGTACC ATGGAAGCTT      1380
 50   ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC CGAAAGGAA GCTGAGTTGG      1440
      CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG GGCCTCTAAA CGGGTCTTGA      1500
```

GGGGTTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT TCACGC   1546

(2) INFORMATION FOR SEQ ID NO:51:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 47 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TATGAAACAT CATCACCATC ACCATCATGC TAGCGTTAAC GCGTTGG   47

(2) INFORMATION FOR SEQ ID NO:52:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 49 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

AATTCCAACG CGTTAACGCT AGCATGATGG TGATGGTGAT GATGTTTCA   49

(2) INFORMATION FOR SEQ ID NO:53:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 141 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

- 165 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CTAATTCCGC TCTCACCTAC CAAACAATGC CCCCCTGCAA AAAATAAATT CATATAAAAA   60

ACATACAGAT AACCATCTGC GGTGATAAAT TATCTCTGGC GGTGTTGACA TAAATACCAC   120

TGGCGGTGAT ACTGAGCACA T   141

(2) INFORMATION FOR SEQ ID NO:54:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 147 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CGATGTGCTC AGTATCACCG CCAGTGGTAT TTATGTCAAC ACCGCCAGAG ATAATTTATC   60

ACCGCAGATG GTTATCTGTA TGTTTTTTAT ATGAATTTAT TTTTTGCAGG GGGGCATTGT   120

TTGGTAGGTG AGAGCGGAAT TAGACGT   147

(2) INFORMATION FOR SEQ ID NO:55:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 55 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGGAATTC GGTAC   55

- 166 -

(2) INFORMATION FOR SEQ ID NO:56:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 49 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CGAATTCCAA CGCGTTAACC ATATGTTATT CCTCCTTCTA GAATCAAAT   49

(2) INFORMATION FOR SEQ ID NO:57:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 668 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GTGAAGAGCG TGAAGAGCGG TTCCTCCTTT CAGCAAAAAA CCCCTCAAGA CCCGTTTAGA   60

GGCCCCAAGG GGTTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAACT CAGCTTCCTT   120

TCGGGCTTTC TTCTTCTTCT TCTTCTTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC   180

CTGCAGGTCG ACACTAGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCTCCTT   240

TAATTAGTTA ACTCAAATCT AGAATCAAAT CGATAAATTG TGAGCGCTCA CAATTGAGAA   300

TATTAATCAA GAATTTTAGC ATTTGTCAAA TGAATTTTTT AAAAATTATG AGACGTCCAT   360

ATTTGAATGT ATTTAGAAAA ATAAACAAAA GAGTTTGTAG AAACGCAAAA AGGCCATCCG   420

TCAGGATGGC CTTCTGCTTA ATTTGATGCC TGGCAGTTTA TGGCGGGCGT CCTGCCCGCC   480

ACCCTCCGGG CCGTTGCTTC GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCCTACTCA   540

GGAGAGCGTT CACCGACAAA CAACAGATAA AACGAAAGGC CCAGTC TC GACTGAGCCT   600

- 167 -

```
TTCGTTTTAT TTGATGCCTG GCAGTTCCCT ACTCTCGCAT GGGGAGACCA TGCATACGTT        660

ACGCACGT                                                                  668
```

(2) INFORMATION FOR SEQ ID NO:58:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 726 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

```
GCGTAACGTA TGCATGGTCT CCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA         60

CGAAAGGCTC AGTCGAAAGA CTGGGCCTTT CGTTTTATCT GTTGTTTGTC GGTGAACGCT        120

CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG TTGCGAAGCA ACGGCCCGGA        180

GGGTGGCGGG CAGGACGCCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGGGCCT        240

CCCACCGCCC GTCCTGCGGG CGGTATTTGA CGGTCCGTAG TTTAATTCGT CTTCGCCATC        300

CTGACGGATG GCCTTTTTGC GTTTCTACAA ACTCTTTTGT TTATTTTTCT AAATACATTC        360

AAATATGGAC GTCTCATAAT TTTTAAAAAA TTCATTTGAC AAATGCTAAA ATTCTTGATT        420

AATATTCTCA ATTGTGAGCG CTCACAATTT ATCGATTTGA TTCTAGATTT GTTTTAACTA        480

ATTAAAGGAG GAATAACATA TGGTTAACGC GTTGGAATTC GAGCTCACTA GTGTCGACCT        540

GCAGGGTACC ATGGAAGCTT ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC        600

CCGAAAGGAA GCTGAGTTGG CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG        660

GGCCTCTAAA CGGGTCTTGA GGGGTTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT        720

TCACGC                                                                   726
```

- 168 -

(2) INFORMATION FOR SEQ ID NO:59:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 44 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT GGAC   44

(2) INFORMATION FOR SEQ ID NO:60:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 27 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GTCCTCCTGG TACCTACCTA AAACAAC   27

(2) INFORMATION FOR SEQ ID NO:61:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 102 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTACCCGGCG   60

GACATTTATC ACACAGCAGC TGATGAGAAG TTTCTTCATC CA   102

- 169 -

(2) INFORMATION FOR SEQ ID NO:62:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

    Met Asp Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro
      1   5   10   15

    Gly Thr Tyr

(2) INFORMATION FOR SEQ ID NO:63:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 84 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TATGGAAACT TTTCCTCCAA AATATCTTCA TTATGATGAA GAAACTTCTC ATCAGCTGCT   60

GTGTGATAAA TGTCCGCCGG GTAC   84

(2) INFORMATION FOR SEQ ID NO:64:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 78 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 170 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCATAA TGAAGATATT   60

TTGGAGGAAA AGTTTCCA   78

(2) INFORMATION FOR SEQ ID NO:65:

     (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 44 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTCACGGATT GAAC   44

(2) INFORMATION FOR SEQ ID NO:66:

     (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 38 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

GTGCTCCTGG TACCTACCTA AAACAGCACT GCACAGTG   38

(2) INFORMATION FOR SEQ ID NO:67:

     (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 84 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

- 171 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATGGAAACT CTGCCTCCAA AATACCTGCA TTACGATCCG GAAACTGGTC ATCAGCTGCT   60

GTGTGATAAA TGTGCTCCGG GTAC   84

(2) INFORMATION FOR SEQ ID NO:68:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 78 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT   60

TTGGAGGCAG AGTTTCCA   78

(2) INFORMATION FOR SEQ ID NO:69:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 54 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TATGGACCCA GAAACTGGTC ATCAGCTGCT GTGTGATAAA TGTGCTCCGG GTAC   54

(2) INFORMATION FOR SEQ ID NO:70:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 48 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

- 172 -

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC TGGGTCCA   48

(2) INFORMATION FOR SEQ ID NO:71:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 87 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

TATGAAAGAA ACTCTGCCTC CAAAATACCT GCATTACGAT CCGGAAACTG GTCATCAGCT   60

GCTGTGTGAT AAATGTGCTC CGGGTAC   87

(2) INFORMATION FOR SEQ ID NO:72:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 81 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT   60

TTGGAGGCAG AGTTTCTTTC A   81

- 173 -

(2) INFORMATION FOR SEQ ID NO:73:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 71 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GTTCTCCTCA TATGAAACAT CATCACCATC ACCATCATGA AACTCTGCCT CCAAAATACC   60

TGCATTACGA T   71

(2) INFORMATION FOR SEQ ID NO:74:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 43 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GTTCTCCTCA TATGAAAGAA ACTCTGCCTC CAAAATACCT GCA   43

(2) INFORMATION FOR SEQ ID NO:75:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 76 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 174 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TACGCACTGG ATCCTTAATG ATGGTGATGG TGATGATGTA AGCAGCTTAT TTTCACGGAT   60

TGAACCTGAT TCCCTA   76

(2) INFORMATION FOR SEQ ID NO:76:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 47 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GTTCTCCTCA TATGAAATAC CTGCATTACG ATCCGGAAAC TGGTCAT   47

(2) INFORMATION FOR SEQ ID NO:77:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 43 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

GTTCTCCTAT TAATGAAATA TCTTCATTAT GATGAAGAAA CTT   43

(2) INFORMATION FOR SEQ ID NO:78:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 40 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 175 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

5   TACGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT   40

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
10   (A) LENGTH: 40 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

15   (ii) MOLECULE TYPE: cDNA

20   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

GTTCTCCTCA TATGGAAACT CTGCCTCCAA AATACCTGCA   40

(2) INFORMATION FOR SEQ ID NO:80:
25
(i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 43 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
30   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TACGCACTGG ATCCTTATGT TGCATTTCCT TTCTGAATTA GCA   43
40
(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 18 base pairs
45   (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
50

- 176 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

CCGGAAACAG ATAATGAG   18

(2) INFORMATION FOR SEQ ID NO:82:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 18 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GATCCTCATT ATCTGTTT   18

(2) INFORMATION FOR SEQ ID NO:83:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CCGGAAACAG AGAAGCCACG CAAAAGTAAG   30

(2) INFORMATION FOR SEQ ID NO:84:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 177 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

GATCCTTACT TTTGCGTGGC TTCTCTGTTT   30

(2) INFORMATION FOR SEQ ID NO:85:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATGTTAATG AG   12

(2) INFORMATION FOR SEQ ID NO:86:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

GATCCTCATT AACA   14

(2) INFORMATION FOR SEQ ID NO:87:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 173 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

5   TATGTTCCGG AAACAGTTAA G   21

(2) INFORMATION FOR SEQ ID NO:88:

        (i) SEQUENCE CHARACTERISTICS:
10   (A) LENGTH: 23 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

15   (ii) MOLECULE TYPE: cDNA

20

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

       GATCCTTAAC TGTTTCCGGA ACA   23
25
       (2) INFORMATION FOR SEQ ID NO:89:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 36 base pairs
30   (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: cDNA
35

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:
40
       TATGTTCCGG AAACAGTGAA TCAACTCAAA AATAAG   36

       (2) INFORMATION FOR SEQ ID NO:90:

45   (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 38 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear
50
        (ii) MOLECULE TYPE: cDNA

- 179 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GATCCTTATT TTTGAGTTGA TTCACTGTTT CCGGAACA   38

(2) INFORMATION FOR SEQ ID NO:91:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 100 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

CTAGCGACGA CGACGACAAA GAAACTCTGC CTCCAAAATA CCTGCATTAC GATCCGGAAA   60

CTGGTCATCA GCTGCTGTGT GATAAATGTG CTCCGGGTAC   100

(2) INFORMATION FOR SEQ ID NO:92:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 92 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT   60

TTGGAGGCAG AGTTTCTTTG TCGTCGTCGT CG   92

- 130 -

(2) INFORMATION FOR SEQ ID NO:93:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 26 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: cDNA

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ACAAACACAA TCGATTTGAT ACTAGA   26

(2) INFORMATION FOR SEQ ID NO:94:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 50 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: cDNA

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TTTGTTTTAA CTAATTAAAG GAGGAATAAA ATATGAGAGG ATCGCATCAC   50

(2) INFORMATION FOR SEQ ID NO:95:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 50 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: cDNA

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

CATCACCATC ACGAAACCTT CCCGCCGAAA TACCTGCACT ACGACGAAGA   50

- 181 -

(2) INFORMATION FOR SEQ ID NO:96:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 49 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

AACCTCCCAC CAGCTGCTGT GCGACAAATG CCCGCCGGGT ACCCAAACA   49

(2) INFORMATION FOR SEQ ID NO:97:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

TGTTTGGGTA CCCGGCGGGC ATTTGT   26

(2) INFORMATION FOR SEQ ID NO:98:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 50 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CGCACAGCAG CTGGTGGGAG GTTTCTTCGT CGTAGTGCAG GTATTTCGGC   50

(2) INFORMATION FOR SEQ ID NO:99:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 49 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GGGAAGGTTT CGTGATGGTG ATGGTGATGC GATCCTCTCA TATTTTATT   49

(2) INFORMATION FOR SEQ ID NO:100:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 50 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

CCTCCTTTAA TTAGTTAAAA CAAATCTAGT ATCAAATCGA TTGTGTTTGT   50

(2) INFORMATION FOR SEQ ID NO:101:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 59 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

ACAAACACAA TCGATTTGAT ACTAGATTTG TTTTAACTAA TTAAAGGAGG AATAAAATG   59

- 185 -

(2) INFORMATION FOR SEQ ID NO:102:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 48 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

CTAATTAAAG GAGGAATAAA ATGAAAGAAA CTTTTCCTCC AAAATATC   48

(2) INFORMATION FOR SEQ ID NO:103:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C   31

(2) INFORMATION FOR SEQ ID NO:104:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 59 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

ACAAACACAA TCGATTTGAT ACTAGATTTG TTTTAACTAA TTAAAGGAGG AATAAAATG   59

- 184 -

(2) INFORMATION FOR SEQ ID NO:105:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

CTAATTAAAG GAGGAATAAA ATGAAAAAAA AAGAAACTTT TCCTCCAAAA TATC   54

(2) INFORMATION FOR SEQ ID NO:106:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C   31

(2) INFORMATION FOR SEQ ID NO:107:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 44 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

CAGCCCGGGT AAAATGGAAA CGTTTCCTCC AAAATATCTT CATT   44

- 135 -

(2) INFORMATION FOR SEQ ID NO:108:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 44 base pairs
5   (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA
10

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:
15

CGTTTCCATT TTACCCGGGC TGAGCGAGAG GCTCTTCTGC GTGT   44

(2) INFORMATION FOR SEQ ID NO:109:

20   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
25

    (ii) MOLECULE TYPE: cDNA

30

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

CGCTCAGCCC GGGTAAAATG GAAACGTTGC CTCCAAAATA CCTGC   45

35 (2) INFORMATION FOR SEQ ID NO:110:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 39 base pairs
        (B) TYPE: nucleic acid
40   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

45

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

50 CCATTTTACC CGGGCTGAGC GAGAGGCTCT TCTGCGTGT   39

- 186 -

(2) INFORMATION FOR SEQ ID NO:111:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 36 base pairs
5   (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA
10

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:
15

GAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC   36

(2) INFORMATION FOR SEQ ID NO:112:

20   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 34 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
25

    (ii) MOLECULE TYPE: cDNA

30

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

CAGCTGCAGC TAAGCAGCTT ATTTTCACGG ATTG   34

35 (2) INFORMATION FOR SEQ ID NO:113:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 36 base pairs
       (B) TYPE: nucleic acid
40   (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

45

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

50 AAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC   36

- 137 -

(2) INFORMATION FOR SEQ ID NO:114:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

CAGCTGCAGC TAAGCAGCTT ATTTTTACTG ATTGG   35

(2) INFORMATION FOR SEQ ID NO:115:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 102 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

CTAGAAGGAG GAATAACATA TGGAAACTTT TGCTCCAAAA TATCTTCATT ATGATGAAGA   60

AACTAGTCAT CAGCTGCTGT GTGATAAATG TCCGCCGGGT AC   102

(2) INFORMATION FOR SEQ ID NO:116:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 94 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

```
CCGGCGGACA TTTATCACAC AGCAGCTGAT GACTAGTTTC TTCATCATAA TGAAGATATT      60
```

5   TTGGAGCAAA AGTTTCCATA TGTTATTCCT CCTT

                                                                 94

(2) INFORMATION FOR SEQ ID NO:117:

10   (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 62 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

15   (ii) MOLECULE TYPE: cDNA

20   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

```
CTAGAAGGAG GAATAACATA TGGAAACTTT TCCTGCTAAA TATCTTCATT ATGATGAAGA      60
```

   AA

25   62

(2) INFORMATION FOR SEQ ID NO:118:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 62 base pairs
30   (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA
35

40   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

```
CTAGTTTCTT CATCATAATG AAGATATTTA GCAGGAAAAG TTTCCATATG TTATTCCTCC      60
```

   TT

                                                               62

45   (2) INFORMATION FOR SEQ ID NO:119:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 51 amino acids
             (B) TYPE: amino acid
50   (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

- 199 -

```
        (ii) MOLECULE TYPE: protein


  5

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:


      Tyr His Tyr Tyr Asp Gln Asn Gly Arg Met Cys Glu Glu Cys His Met
      1               5                   10                  15
 10

      Cys Gln Pro Gly His Phe Leu Val Lys His Cys Lys Gln Pro Lys Arg
                  20                  25                  30


      Asp Thr Val Cys His Lys Pro Cys Glu Pro Gly Val Thr Tyr Thr Asp
 15               35                  40                  45


      Asp Trp His
          50

 20  (2) INFORMATION FOR SEQ ID NO:120:


        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 2432 base pairs
            (B) TYPE: nucleic acid
 25         (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

 30
        (ix) FEATURE:
            (A) NAME/KEY: CDS
            (B) LOCATION: 124..1326

 35
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

     ATCAAAGGCA GGGCATACTT CCTGTTGCCC AGACCTTATA TAAAACGTCA TGTTCGCCTG       60

 40  GGCAGCAGAG AAGCACCTAG CACTGGCCCA GCGGCTGCCG CCTGAGGTTT CCAGAGGACC      120

     ACA ATG AAC AAG TGG CTG TGC TGT GCA CTC CTG GTG TTC TTG GAC ATC       168
         Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile
         1               5                   10                  15
 45
     ATT GAA TGG ACA ACC CAG GAA ACC TTT CCT CCA AAA TAC TTG CAT TAT       216
     Ile Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr
                     20                  25                  30

 50
```

- 190 -

```
      GAC CCA GAA ACC GGA CGT CAG CTC TTG TGT GAC AAA TGT GCT CCT GGC        264
      Asp Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly
                   35                40                45

  5   ACC TAC CTA AAA CAG CAC TGC ACA GTC AGG AGG AAG ACA CTG TGT GTC        312
      Thr Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val
                   50                55                60

      CCT TGC CCT GAC TAC TCT TAT ACA GAC AGC TGG CAC ACG AGT GAT GAA        360
 10   Pro Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu
                   65                70                75

      TGC GTG TAC TGC AGC CCC GTG TGC AAG GAA CTG CAG ACC GTG AAA CAG        408
 15   Cys Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln
           80                85                90                95

      GAG TGC AAC CGC ACC CAC AAC CGA GTG TGC GAA TGT GAG GAA GGG CGC        456
      Glu Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg
                  100               105               110
 20

      TAC CTG GAG CTC GAA TTC TGC TTG AAG CAC CGG AGC TGT CCC CCA GGC        504
      Tyr Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly
                  115               120               125

 25   TTG GGT GTG CTG CAG GCT GGG ACC CCA GAG CGA AAC ACG GTT TGC AAA        552
      Leu Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys
                  130               135               140

      AGA TGT CCG GAT GGG TTC TTC TCA GGT GAG ACG TCA TCG AAA GCA CCC        600
 30   Arg Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro
           145               150               155

      TGT AGG AAA CAC ACC AAC TGC AGC TCA CTT GGC CTC CTG CTA ATT CAG        648
      Cys Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Leu Ile Gln
 35   160               165               170               175

      AAA GGA AAT GCA ACA CAT GAC AAT GTA TGT TCC GGA AAC AGA GAA GCA        696
      Lys Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala
                  180               185               190
 40

      ACT CAA AAT TGT GGA ATA GAT GTC ACC CTG TGC GAA GAG GCA TTC TTC        744
      Thr Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe
                  195               200               205

 45   AGG TTT GCT GTG CCT ACC AAG ATT ATA CCG AAT TGG CTG AGT GTT CTG        792
      Arg Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu
                  210               215               220

      GTG GAC AGT TTG CCT GGG ACC AAA GTG AAT GCA GAG AGT GTA GAG AGG        840
 50   Val Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg
                  225               230               235
```

- 191 -

```
ATA AAA CGG AGA CAC AGC TCG CAA GAG CAA ACT TTC CAG CTA CTT AAG    888
Ile Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys
240             245             250             255
```

```
CTG TGG AAG CAT CAA AAC AGA GAC CAG GAA ATG GTG AAG AAG ATC ATC    936
Leu Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile
260             265             270
```

```
CAA GAC ATT GAC CTC TGT GAA AGC AGT GTG CAA CGG CAT ATC GGC CAC    984
Gln Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His
275             280             285
```

```
GCG AAC CTC ACC ACA GAG CAG CTC CGC ATC TTG ATG GAG AGC TTG CCT    1032
Ala Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro
290             295             300
```

```
GGG AAG AAG ATC AGC CCA GAC GAG ATT GAG AGA ACG AGA AAG ACC TGC    1080
Gly Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys
305             310             315
```

```
AAA CCC AGC GAG CAG CTC CTG AAG CTA CTG AGC TTG TGG AGG ATC AAA    1128
Lys Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys
320             325             330             335
```

```
AAT GGA GAC CAA GAC ACC TTG AAG GGC CTG ATG TAC GCA CTC AAG CAC    1176
Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His
340             345             350
```

```
TTG AAA GCA TAC CAC TTT CCC AAA ACC GTC ACC CAC AGT CTG AGG AAG    1224
Leu Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys
355             360             365
```

```
ACC ATC AGG TTC TTG CAC AGC TTC ACC ATG TAC CGA TTG TAT CAG AAA    1272
Thr Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys
370             375             380
```

```
CTC TTT CTA GAA ATG ATA GGG AAT CAG GTT CAA TCA GTG AAG ATA AGC    1320
Leu Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser
385             390             395
```

```
TGC TTA TAGTTAGGAA TGGTCACTGG CTGTTTCTT CAGGATGGGC CAACACTGAT    1376
Cys Leu
400
```

```
GGAGCAGATG CTGCTTCTC CGGCTCTTGA AATGGCAGTT GATTCCTTTC TCATCAGTTG    1436
```

```
GTGGGAATGA AGATCCTCCA GCCCAACACA CACACTGGGG AGTCTGAGTC AGGAGAGTGA    1496
```

```
GGCAGGCTAT TTGATAATTG TGCAAAGCTG CCAGGTGTAC ACCTAGAAAG TCAAGCACCC    1556
```

- 192 -

```
TGAGAAAGAG GATATTTTTA TAACCTCAAA CATAGGCCCT TTCCTTCCTC TCCTTATGGA      1616

TGAGTACTCA GAAGGCTTCT ACTATCTTCT GTGTCATCCC TAGATGAAGG CCTCTTTTAT      1676

TTATTTTTT ATTCTTTTTT TCGGAGCTGG GGACCGAACC CAGGGCCTTG CGCTTGCGAG       1736

GCAAGTGCTC TACCACTGAG CTAAATCTCC AACCCCTGAA GGCCTCTTTC TTTCTGCCTC      1796

TGATAGTCTA TGACATTCTT TTTTCTACAA TTCGTATCAG GTGCACGAGC CTTATCCCAT      1856

TTGTAGGTTT CTAGGCAAGT TGACCGTTAG CTATTTTTCC CTCTGAAGAT TTGATTCGAG      1916

TTGCAGACTT GGCTAGACAA GCAGGGGTAG GTTATGGTAG TTTATTTAAC AGACTGCCAC      1976

CAGGAGTCCA GTGTTTCTTG TTCCTCTGTA GTTGTACCTA AGCTGACTCC AAGTACATTT      2036

AGTATGAAAA ATAATCAACA AATTTTATTC CTTCTATCAA CATTGGCTAG CTTTGTTTCA      2096

GGGCACTAAA AGAAACTACT ATATGGAGAA AGAATTGATA TTGCCCCCAA CGTTCAACAA      2156

CCCAATAGTT TATCCAGCTG TCATGCCTGG TTCAGTGTCT ACTGACTATG CGCCCTCTTA      2216

TTACTGCATG CAGTAATTCA ACTGGAAATA GTAATAATAA TAATAGAAAT AAAATCTAGA      2276

CTCCATTGGA TCTCTCTGAA TATGGGAATA TCTAACTTAA GAAGCTTTGA GATTTCAGTT      2336

GTGTTAAAGG CTTTTATTAA AAAGCTGATG CTCTTCTGTA AAAGTTACTA ATATATCTGT      2396

AAGACTATTA CAGTATTGCT ATTTATATCC ATCCAG                                2432
```

(2) INFORMATION FOR SEQ ID NO:121:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 401 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

```
Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile
 1               5                  10                  15

Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
            20                  25                  30
```

- 193 -

```
          Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
                   35                  40                  45

  5   Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
              50                  55                  60

      Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
          65              70              75                  80
 10
      Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu
                      85                  90                  95

      Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
 15                 100             105                 110

      Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu
                  115                 120                 125

 20   Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
              130                 135                 140

      Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
          145             150                 155                 160
 25
      Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Leu Ile Gln Lys
                      165                 170                 175

      Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
 30                 180             185                 190

      Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
                  195                 200                 205

 35   Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val
              210                 215                 220

      Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
          225             230                 235                 240
 40
      Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
                      245                 250                 255

      Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln
 45                 260             265                 270

      Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His Ala
          275             280                 285

 50   Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro Gly
          290                 295                 300
```

- 194 -

```
        Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys
        305             310             315             320

  5     Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
                        325             330             335

        Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu
                    340             345             350
 10
        Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr
                    355             360             365

        Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu
 15         370             375             380

        Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
        385             390             395             400
 20     Leu
```

(2) INFORMATION FOR SEQ ID NO:122:

 25   (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 1324 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear
 30
            (ii) MOLECULE TYPE: cDNA

 35   (ix) FEATURE:
                (A) NAME/KEY: CDS
                (B) LOCATION: 90..1292

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:
 40
```
CCTTATATAA ACGTCATGAT TGCCTGGGCT GCAGAGACGC ACCTAGCACT GACCCAGCGG          60

CTGCCTCCTG AGGTTTCCCG AGGACCACA ATG AAC AAG TGG CTG TGC TGC GCA          113
                                 Met Asn Lys Trp Leu Cys Cys Ala
                                  1                   5

 45

CTC CTG GTG CTC CTG GAC ATC ATT GAA TGG ACA ACC CAG GAA ACC CTT          161
Leu Leu Val Leu Leu Asp Ile Ile Glu Trp Thr Thr Gln Glu Thr Leu
 50     10                  15                  20
```

- 195 -

```
        CCT CCA AAG TAC TTG CAT TAT GAC CCA GAA ACT GGT CAT CAG CTC CTG      209
        Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His Gln Leu Leu
         25              30              35              40

   5    TGT GAC AAA TGT GCT CCT GGC ACC TAC CTA AAA CAG CAC TGC ACA GTG      257
        Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His Cys Thr Val
                     45              50              55

        AGG AGG AAG ACA TTG TGT GTC CCT TGC CCT GAC CAC TCT TAT ACG GAC      305
  10    Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser Tyr Thr Asp
                     60              65              70

        AGC TGG CAC ACC AGT GAT GAG TGT GTG TAT TGC AGC CCA GTG TGC AAG      353
        Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro Val Cys Lys
  15             75              80              85

        GAA CTG CAG TCC GTG AAG CAG GAG TGC AAC CGC ACC CAC AAC CGA GTG      401
        Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val
                90              95             100
  20
        TGT GAG TGT GAG GAA GGG CGT TAC CTG GAG ATC GAA TTC TGC TTG AAG      449
        Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu Lys
        105             110             115             120

  25    CAC CGG AGC TGT CCC CCG GGC TCC GGC GTG GTG CAA GCT GGA ACC CCA      497
        His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala Gly Thr Pro
                     125             130             135

        GAG CGA AAC ACA GTT TGC AAA AAA TGT CCA GAT GGG TTC TTC TCA GGT      545
  30    Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe Phe Ser Gly
                     140             145             150

        GAG ACT TCA TCG AAA GCA CCC TGT ATA AAA CAC ACG AAC TGC AGC ACA      593
        Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn Cys Ser Thr
  35             155             160             165

        TTT GGC CTC CTG CTA ATT CAG AAA GGA AAT GCA ACA CAT GAC AAC GTG      641
        Phe Gly Leu Leu Leu Ile Gln Lys Gly Asn Ala Thr His Asp Asn Val
                170             175             180
  40
        TGT TCC GGA AAC AGA GAA GCC ACG CAA AAG TGT GGA ATA GAT GTC ACC      689
        Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile Asp Val Thr
        185             190             195             200

  45    CTG TGT GAA GAG GCC TTC TTC AGG TTT GCT GTT CCT ACC AAG ATT ATA      737
        Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Ile Ile
                     205             210             215


  50
```

- 196 -

```
    CCA AAT TGG CTG AGT GTT TTG GTG GAC AGT TTG CCT GGG ACC AAA GTG          735
    Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly Thr Lys Val
                220                     225                 230

 5  AAT GCC GAG AGT GTA GAG AGG ATA AAA CGG AGA CAC AGC TCA CAA GAG          833
    Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser Ser Gln Glu
                235                     240                 245

    CAA ACC TTC CAG CTG CTG AAG CTG TGG AAA CAT CAA AAC AGA GAC CAG          381
10  Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Arg Asp Gln
                250                     255                 260

    GAA ATG GTG AAG AAG ATC ATC CAA GAC ATT GAC CTC TGT GAA AGC AGC          929
    Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Ser Ser
15  265                     270                 275                 280

    GTG CAG CGG CAT CTC GGC CAC TCG AAC CTC ACC ACA GAG CAG CTT CTT          977
    Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu Gln Leu Leu
                285                     290                 295
20
    GCC TTG ATG GAG AGC CTG CCT GGG AAG AAG ATC AGC CCA GAA GAG ATT         1025
    Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro Glu Glu Ile
                300                     305                 310

25  GAG AGA ACG AGA AAG ACC TGC AAA TCG AGC GAG CAG CTC CTG AAG CTA         1073
    Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu Leu Lys Leu
                315                     320                 325

    CTC AGT TTA TGG AGG ATC AAA AAT GGT GAC CAA GAC ACC TTG AAG GGC         1121
30  Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly
                330                     335                 340

    CTG ATG TAT GCC CTC AAG CAC TTG AAA ACA TCC CAC TTT CCC AAA ACT         1169
    Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe Pro Lys Thr
35  345                     350                 355                 360

    GTC ACC CAC AGT CTG AGG AAG ACC ATG AGG TTC CTG CAC AGC TTC ACA         1217
    Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His Ser Phe Thr
                365                     370                 375
40
    ATG TAC AGA CTG TAT CAG AAG CTC TTT TTA GAA ATG ATA GGG AAT CAG         1265
    Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn Gln
                380                     385                 390

45  GTT CAA TCC GTG AAA ATA AGC TGC TTA TAACTAGGAA TGGTCACTGG              1312
    Val Gln Ser Val Lys Ile Ser Cys Leu
                395                     400


    GCTGTTTCTT CA                                                           1324
50
```

- 197 -

(2) INFORMATION FOR SEQ ID NO:123:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 401 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

```
Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Leu Leu Asp Ile Ile
 1               5                  10                  15

Glu Trp Thr Thr Gln Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp
            20                  25                  30

Pro Glu Thr Gly His Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
            35                  40                  45

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
        50                  55                  60

Cys Pro Asp His Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
    65                  70                  75                  80

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu
                85                  90                  95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
            100                 105                 110

Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser
            115                 120                 125

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys
        130                 135                 140

Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
    145                 150                 155                 160

Ile Lys His Thr Asn Cys Ser Thr Phe Gly Leu Leu Leu Ile Gln Lys
                165                 170                 175

Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
            180                 185                 190

Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
            195                 200                 205
```

- 198 -

```
      Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val
          210               215               220

  5   Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
      225               230               235               240

      Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
                    245               250               255

 10   Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln
                    260               265               270

      Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Leu Gly His Ser
 15               275               280               285

      Asn Leu Thr Thr Glu Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly
                290               295               300

 20   Lys Lys Ile Ser Pro Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys
      305               310               315               320

      Ser Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
                    325               330               335

 25   Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu
                340               345               350

      Lys Thr Ser His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr
 30               355               360               365

      Met Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu
                370               375               380

 35   Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
      385               390               395               400

      Leu

 40

      (2) INFORMATION FOR SEQ ID NO:124:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 1355 base pairs
 45           (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: cDNA
 50
```

- 199 -

```
(ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 94..1296
```

```
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:
```

```
GTATATATAA CGTGATGAGC GTACGGGTGC GGAGACGCAC CGGAGCGCTC GCCCAGCCGC      60

CGCTCCAAGC CCCTGAGGTT TCCGGGGACC ACA ATG AAC AAG TTG CTG TGC TGC     114.
                                      Met Asn Lys Leu Leu Cys Cys
                                       1               5

GCG CTC GTG TTT CTG GAC ATC TCC ATT AAG TGG ACC ACC CAG GAA ACG      162
Ala Leu Val Phe Leu Asp Ile Ser Ile Lys Trp Thr Thr Gln Glu Thr
        10              15              20

TTT CCT CCA AAG TAC CTT CAT TAT GAC GAA GAA ACC TCT CAT CAG CTG      210
Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His Gln Leu
        25              30              35

TTG TGT GAC AAA TGT CCT CCT GGT ACC TAC CTA AAA CAA CAC TGT ACA      258
Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His Cys Thr
    40              45              50                  55

GCA AAG TGG AAG ACC GTG TGC GCC CCT TGC CCT GAC CAC TAC TAC ACA      306
Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr
                60                  65                  70

GAC AGC TGG CAC ACC AGT GAC GAG TGT CTA TAC TGC AGC CCC GTG TGC      354
Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys
                75                  80                  85

AAG GAG CTG CAG TAC GTC AAG CAG GAG TGC AAT CGC ACC CAC AAC CGC      402
Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg
            90                  95                  100

GTG TGC GAA TGC AAG GAA GGG CGC TAC CTT GAG ATA GAG TTC TGC TTG      450
Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu
        105                 110                 115

AAA CAT AGG AGC TGC CCT CCT GGA TTT GGA GTG GTG CAA GCT GGA ACC      498
Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala Gly Thr
    120                 125                 130                 135

CCA GAG CGA AAT ACA GTT TGC AAA AGA TGT CCA GAT GGG TTC TTC TCA      546
Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe Phe Ser
                140                 145                 150
```

- 200 -

```
AAT GAG ACG TCA TCT AAA GCA CCC TGT AGA AAA CAC ACA AAT TGC AGT      594
Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn Cys Ser
        155                 160                 165
```

5
```
GTC TTT GGT CTC CTG CTA ACT CAG AAA GGA AAT GCA ACA CAC GAC AAC      642
Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His Asp Asn
        170                 175                 180
```

10
```
ATA TGT TCC GGA AAC AGT GAA TCA ACT CAA AAA TGT GGA ATA GAT GTT      690
Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile Asp Val
        185                 190                 195
```

15
```
ACC CTG TGT GAG GAG GCA TTC TTC AGG TTT GCT GTT CCT ACA AAG TTT      738
Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Phe
200                 205                 210                 215
```

```
ACG CCT AAC TGG CTT AGT GTC TTG GTA GAC AAT TTG CCT GGC ACC AAA      786
Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly Thr Lys
20                  220                 225                 230
```

```
GTA AAC GCA GAG AGT GTA GAG AGG ATA AAA CGG CAA CAC AGC TCA CAA      834
Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser Ser Gln
25                  235                 240                 245
```

```
GAA CAG ACT TTC CAG CTG CTG AAG TTA TGG AAA CAT CAA AAC AAA GCC      882
Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Lys Ala
        250                 255                 260
30
```

```
CAA GAT ATA GTC AAG AAG ATC ATC CAA GAT ATT GAC CTC TGT GAA AAC      930
Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Asn
        265                 270                 275
```

35
```
AGC GTG CAG CGG CAC ATT GGA CAT GCT AAC CTC ACC TTC GAG CAG CTT      978
Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu Gln Leu
280                 285                 290                 295
```

```
CGT AGC TTG ATG GAA AGC TTA CCG GGA AAG AAA GTG GGA GCA GAA GAC      1026
Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp
40                  300                 305                 310
```

```
ATT GAA AAA ACA ATA AAG GCA TGC AAA CCC AGT GAC CAG ATC CTG AAG      1074
Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys
45                  315                 320                 325
```

```
CTG CTC AGT TTG TGG CGA ATA AAA AAT GGC GAC CAA GAC ACC TTG AAG      1122
Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys
        330                 335                 340
50
```

- 201 -

```
    GGC CTA ATG CAC GCA CTA AAG CAC TCA AAG ACG TAC CAC TTT CCC AAA    1170
    Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys
        345             350             355

5   ACT GTC ACT CAG AGT CTA AAG AAG ACC ATC AGG TTC CTT CAC AGC TTC    1218
    Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe
        360             365             370             375

    ACA ATG TAC AAA TTG TAT CAG AAG TTA TTT TTA GAA ATG ATA GGT AAC    1266
10  Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn
                    380             385             390

    CAG GTC CAA TCA GTA AAA ATA AGC TGC TTA TAACTGGAAA TGGCCATTGA       1316
    Gln Val Gln Ser Val Lys Ile Ser Cys Leu
15                  395             400

    GCTGTTTCCT CACAATTGGC GAGATCCCAT GGATGATAA                          1355
```

20   (2) INFORMATION FOR SEQ ID NO:125:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 401 amino acids
             (B) TYPE: amino acid
25   (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: protein

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:
30
    Met Asn Lys Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser Ile
     1   5   10   15

    Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
35   20   25   30

    Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr
                35   40   45

40 Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro
            50   55   60

    Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
45 65   70   75   80

    Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu
                    85   90   95

    Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr
50   100   105   110
```

- 202 -

```
    Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe
            115             120             125

 5  Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
            130             135             140

    Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys
            145             150             155             160

10  Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys
                    165             170             175

    Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr
            180             185             190

15  Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
            195             200             205

    Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val
20          210             215             220

    Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
    225             230             235             240

25  Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
                    245             250             255

    Trp Lys His Gln Asn Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln
            260             265             270

30  Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala
            275             280             285

    Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly
35          290             295             300

    Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys
    305             310             315             320

40  Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
                    325             330             335

    Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser
            340             345             350

45  Lys Thr Tyr His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr
            355             360             365

    Ile Arg Phe Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu
50          370             375             380
```

- 203 -

```
Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
385                 390                 395                 400

Leu
```

(2) INFORMATION FOR SEQ ID NO:126:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 139 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

```
Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys
1               5               10                  15

Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
            20              25              30

Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala
        35              40              45

Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys
    50              55              60

Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr
65              70              75              80

Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn
            85              90              95

Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His
        100             105             110

Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly
        115             120             125

Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys
        130             135
```

- 204 -

(2) INFORMATION FOR SEQ ID NO:127:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 48 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA   48

(2) INFORMATION FOR SEQ ID NO:128:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 219 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

```
Met Leu Gly Ile Trp Thr Leu Leu Pro Leu Val Leu Thr Ser Val Ala
1               5                   10                  15

Arg Leu Ser Ser Lys Ser Val Asn Ala Gln Val Thr Asp Ile Asn Ser
                20              25                  30

Lys Gly Leu Glu Leu Arg Lys Thr Val Thr Thr Val Glu Thr Gln Asn
            35              40                  45

Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
        50                  55                  60

Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro
65              70                  75                  80
```

- 205 -

Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His
              85   90   95

Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly
              100   105   110

Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg
        115   120   125

Cys Lys Pro Asn Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp
    130   135   140

Pro Cys Thr Lys Cys Glu His Gly Ile Ile Lys Glu Cys Thr Leu Thr
145   150   155   160

Ser Asn Thr Lys Cys Lys Glu Glu Gly Ser Arg Ser Asn Leu Gly Trp
              165   170   175

Leu Cys Leu Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg
          180   185   190

Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly
          195   200   205

Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr
    210   215

(2) INFORMATION FOR SEQ ID NO:129:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 280 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
1   5   10   15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
          20   25   30

His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
          35   40   45

- 206 -

```
      Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
          50                  55                  60

  5   Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
          65                  70                  75                  80

      Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
                      85                  90                  95

 10   Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
                  100                 105                 110

      Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
                  115                 120                 125

 15   Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
              130                 135                 140

      Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
 20   145                 150                 155                 160

      Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
                  165                 170                 175

 25

      Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
                  180                 185                 190

      Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
 30               195                 200                 205

      Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
          210                 215                 220

 35   Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
          225                 230                 235                 240

      Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
                  245                 250                 255
 40

      Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
                  260                 265                 270

      Phe Ser Pro Thr Pro Gly Phe Thr
 45               275                 280



 50
```

- 207 -

(2) INFORMATION FOR SEQ ID NO:130:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 207 amino acids
     5   (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein
10

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:
15
     Met Leu Arg Leu Ile Ala Leu Leu Val Cys Val Val Tyr Val Tyr Gly
     1   5   10   15

     Asp Asp Val Pro Tyr Ser Ser Asn Gln Gly Lys Cys Gly Gly His Asp
20   20   25   30

     Tyr Glu Lys Asp Gly Leu Cys Cys Ala Ser Cys His Pro Gly Phe Tyr
             35   40   45

25   Ala Ser Arg Leu Cys Gly Pro Gly Ser Asn Thr Val Cys Ser Pro Cys
         50   55   60

     Glu Asp Gly Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys Val
     65   70   75   80
30
     Ser Cys Arg Gly Pro Cys Thr Gly His Leu Ser Glu Ser Gln Pro Cys
                 85   90   95

     Asp Arg Thr His Asp Arg Val Cys Asn Cys Ser Thr Gly Asn Tyr Cys
35   100   105   110

     Leu Leu Lys Gly Gln Asn Gly Cys Arg Ile Cys Ala Pro Gln Thr Lys
             115   120   125

40   Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Ala Gly Asp Thr
         130   135   140

     Leu Cys Glu Lys Cys Pro Pro His Thr Tyr Ser Asp Ser Leu Ser Pro
     145   150   155   160
45
     Thr Glu Arg Cys Gly Thr Ser Phe Asn Tyr Ile Ser Val Gly Phe Asn
                 165   170   175

     Leu Tyr Pro Val Asn Glu Thr Ser Cys Thr Thr Thr Ala Gly His Asn
50   180   185   190

- 208 -

```
        Glu Val Ile Lys Thr Lys Glu Phe Thr Val Thr Leu Asn Tyr Thr
            195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:131:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 227 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

```
        Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu
        1               5                   10                  15

        Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr
                    20                  25                  30

        Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln
                35                  40                  45

        Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys
            50                  55                  60

        Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp
        65                  70                  75                  80

        Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys
                        85                  90                  95

        Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg
                    100                 105                 110

        Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu
                    115                 120                 125

        Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg
                    130                 135                 140

        Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val
        145                 150                 155                 160

        Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr
                        165                 170                 175
```

- 209 -

```
          Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly
                  180                 185                 190

          Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser
                  195                 200                 205

          Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser
                  210                 215                 220

          Gln His Thr
                  225
```

(2) INFORMATION FOR SEQ ID NO:132:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 197 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

```
          Met Val Ser Leu Pro Arg Leu Cys Ala Leu Trp Gly Cys Leu Leu Thr
          1                 5                 10                  15

          Ala Val His Leu Gly Gln Cys Val Thr Cys Ser Asp Lys Gln Tyr Leu
                  20                  25                  30

          His Asp Gly Gln Cys Cys Asp Leu Cys Gln Pro Gly Ser Arg Leu Thr
                  35                  40                  45

          Ser His Cys Thr Ala Leu Glu Lys Thr Gln Cys His Pro Cys Asp Ser
                  50                  55                  60

          Gly Glu Phe Ser Ala Gln Trp Asn Arg Glu Ile Arg Cys His Gln His
          65                  70                  75                  80

          Arg His Cys Glu Pro Asn Gln Gly Leu Arg Val Lys Lys Glu Gly Thr
                          85                  90                  95

          Ala Glu Ser Asp Thr Val Cys Thr Cys Lys Glu Gly Gln His Cys Thr
                  100                 105                 110

          Ser Lys Asp Cys Glu Ala Cys Ala Gln His Thr Pro Cys Ile Pro Gly
                  115                 120                 125
```

- 210 -

```
Phe Gly Val Met Glu Met Ala Thr Glu Thr Thr Asp Thr Val Cys His
    130             135             140

Pro Cys Pro Val Gly Phe Phe Ser Asn Gln Ser Ser Leu Phe Glu Lys
    145             150             155             160

Cys Tyr Pro Trp Thr Ser Cys Glu Asp Lys Asn Leu Glu Val Leu Gln
                165             170             175

Lys Gly Thr Ser Gln Thr Asn Val Ile Cys Gly Leu Lys Ser Arg Met
                180             185             190

Arg Ala Leu Leu Val
                195
```

(2) INFORMATION FOR SEQ ID NO:133:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 208 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

```
Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile
1               5               10              15

Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
                20              25              30

Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
                35              40              45

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
    50              55              60

Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
    65              70              75              80

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu
                85              90              95
```

- 211 -

        Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
                    100   105   110

5   Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu
                    115   120   125

        Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
                    130   135   140

10   Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
            145   150   155   160

        Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Leu Ile Gln Lys
                        165   170   175

15   Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
                        180   185   190

        Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
20   195   200   205

    (2) INFORMATION FOR SEQ ID NO:134:

25   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 224 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
30
        (ii) MOLECULE TYPE: protein

35
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:

        Met Gly Ala Gly Ala Thr Gly Arg Ala Met Asp Gly Pro Arg Leu Leu
        1   5   10   15
40

        Leu Leu Leu Leu Leu Gly Val Ser Leu Gly Gly Ala Lys Glu Ala Cys
                    20   25   30

45   Pro Thr Gly Leu Tyr Thr His Ser Gly Glu Cys Cys Lys Ala Cys Asn
                    35   40   45

        Leu Gly Glu Gly Val Ala Gln Pro Cys Gly Ala Asn Gln Thr Val Cys
                    50   55   60
50

- 212 -

```
Glu Pro Cys Leu Asp Ser Val Thr Phe Ser Asp Val Val Ser Ala Thr
65                  70                  75                  80

Glu Pro Cys Lys Pro Cys Thr Glu Cys Val Gly Leu Gln Ser Met Ser
                85                  90                  95

Ala Pro Cys Val Glu Ala Asp Asp Ala Val Cys Arg Cys Ala Tyr Gly
            100                 105                 110

Tyr Tyr Gln Asp Glu Thr Thr Gly Arg Cys Glu Ala Cys Arg Val Cys
        115                 120                 125

Glu Ala Gly Ser Gly Leu Val Phe Ser Cys Gln Asp Lys Gln Asn Thr
        130                 135                 140

Val Cys Glu Glu Cys Pro Asp Gly Thr Tyr Ser Asp Glu Ala Asn His
145                 150                 155                 160

Val Asp Pro Cys Leu Pro Cys Thr Val Cys Glu Asp Thr Glu Arg Gln
                165                 170                 175

Leu Arg Glu Cys Thr Arg Trp Ala Asp Ala Glu Cys Glu Glu Ile Pro
                180                 185                 190

Gly Arg Trp Ile Thr Arg Ser Thr Pro Pro Glu Gly Ser Asp Ser Thr
                195             ,   200                 205

Ala Pro Ser Thr Gln Glu Pro Glu Ala Pro Pro Glu Gln Asp Leu Ile
        210                 215                 220
```

(2) INFORMATION FOR SEQ ID NO:135:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 205 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

```
Met Tyr Val Trp Val Gln Gln Pro Thr Ala Phe Leu Leu Leu Gly Leu
1                   5                   10                  15

Ser Leu Gly Val Thr Val Lys Leu Asn Cys Val Lys Asp Thr Tyr Pro
            20                  25                  30
```

- 213 -

```
      Ser Gly His Lys Cys Cys Arg Glu Cys Gln Pro Gly His Gly Met Val
              35            40              45

5     Ser Arg Cys Asp His Thr Arg Asp Thr Val Cys His Pro Cys Glu Pro
              50            55              60

      Gly Phe Tyr Asn Glu Ala Val Asn Tyr Asp Thr Cys Lys Gln Cys Thr
      65            70              75              80
10
      Gln Cys Asn His Arg Ser Gly Ser Glu Leu Lys Gln Asn Cys Thr Pro
                    85              90              95

      Thr Glu Asp Thr Val Cys Gln Cys Arg Pro Gly Thr Gln Pro Arg Gln
15                100             105             110

      Asp Ser Ser His Lys Leu Gly Val Asp Cys Val Pro Cys Pro Pro Gly
                    115             120             125

20    His Phe Ser Pro Gly Ser Asn Gln Ala Cys Lys Pro Trp Thr Asn Cys
              130             135             140

      Thr Leu Ser Gly Lys Gln Ile Arg His Pro Ala Ser Asn Ser Leu Asp
      145             150             155             160
25
      Thr Val Cys Glu Asp Arg Ser Leu Leu Ala Thr Leu Leu Trp Glu Thr
                    165             170             175

      Gln Arg Thr Thr Phe Arg Pro Thr Thr Val Pro Ser Thr Thr Val Trp
30                180             185             190

      Pro Arg Thr Ser Gln Leu Pro Ser Thr Pro Thr Leu Val
              195             200             205
```

35   (2) INFORMATION FOR SEQ ID NO:136:

       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 191 amino acids
           (B) TYPE: amino acid
40   (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: protein

45

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

50   Met Gly Asn Asn Cys Tyr Asn Val Val Val Ile Val Leu Leu Leu Val
       1   5   10   15

- 214 -

```
        Gly Cys Glu Lys Val Gly Ala Val Gln Asn Ser Cys Asp Asn Cys Gln
                  20              25              30

  5     Pro Gly Thr Phe Cys Arg Lys Tyr Asn Pro Val Cys Lys Ser Cys Pro
                  35              40              45

        Pro Ser Thr Phe Ser Ser Ile Gly Gly Gln Pro Asn Cys Asn Ile Cys
                  50              55              60

 10     Arg Val Cys Ala Gly Tyr Phe Arg Phe Lys Lys Phe Cys Ser Ser Thr
        65              70              75              80

        His Asn Ala Glu Cys Glu Cys Ile Glu Gly Phe His Cys Leu Gly Pro
                  85              90              95

 15     Gln Cys Thr Arg Cys Glu Lys Asp Cys Arg Pro Gly Gln Glu Leu Thr
                  100             105             110

        Lys Gln Gly Cys Lys Thr Cys Ser Leu Gly Thr Phe Asn Asp Gln Asn
 20               115             120             125

        Gly Thr Gly Val Cys Arg Pro Trp Thr Asn Cys Ser Leu Asp Gly Arg
                  130             135             140

 25     Ser Val Leu Lys Thr Gly Thr Thr Glu Lys Asp Val Val Cys Gly Pro
        145             150             155             160

        Pro Val Val Ser Phe Ser Pro Ser Thr Thr Ile Ser Val Thr Pro Glu
                  165             170             175

 30     Gly Gly Pro Gly Gly His Ser Leu Gln Val Leu Thr Leu Phe Leu
                  180             185             190
```

35 (2) INFORMATION FOR SEQ ID NO:137:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 54 base pairs
            (B) TYPE: nucleic acid
 40   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

 45

 50   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTAC   54

- 215 -

(2) INFORMATION FOR SEQ ID NO:138:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 380 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:138:

    Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His
    1   5   10   15

    Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr Tyr Leu Lys Gln His
            20   25   30

    Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro Cys Pro Asp His Ser
        35   40   45

    Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Val Tyr Cys Ser Pro
        50   55   60

    Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu Cys Asn Arg Thr His
    65   70   75   80

    Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr Leu Glu Ile Glu Phe
            85   90   95

    Cys Leu Lys His Arg Ser Cys Pro Pro Gly Ser Gly Val Val Gln Ala
            100   105   110

    Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys Cys Pro Asp Gly Phe
            115   120   125

    Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys Ile Lys His Thr Asn
        130   135   140

    Cys Ser Thr Phe Gly Leu Leu Leu Ile Gln Lys Gly Asn Ala Thr His
    145   150   155   160

    Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr Gln Lys Cys Gly Ile
            165   170   175

    Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr
            180   185   190

- 216 -

```
            Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val Asp Ser Leu Pro Gly
                    195                 200                 205

            Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Arg His Ser
                    210                 215                 220

            Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn
            225                 230                 235                 240

            Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys
                            245                 250                 255

            Glu Ser Ser Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu
                            260                 265                 270

            Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro
                    275                 280                 285

            Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu
                    290                 295                 300

            Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr
            305                 310                 315                 320

            Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe
                            325                 330                 335

            Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His
                    340                 345                 350

            Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile
                    355                 360                 365

            Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu
                    370                 375                 380
```

(2) INFORMATION FOR SEQ ID NO:139:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 380 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

- 217 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:

```
          Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His
          1               5                   10                  15
5
          Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His
                        20              25                  30
          Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr
10                  35                  40                  45
          Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro
                    50              55                  60
15        Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His
          65                  70                  75                  80
          Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe
                            85                  90                  95
20
          Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala
                        100             105             110
          Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe
25                  115             120             125
          Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn
                    130             135             140
30        Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His
          145             150             155             160
          Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile
                            165             170             175
35
          Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr
                        180             185             190
          Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly
40                  195             200             205
          Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser
                    210             215             220
45        Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn
          225             230             235             240
          Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys
                            245             250             255
50
```

- 218 -

```
        Glu Asn Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu
                    260                 265                 270

   5    Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala
                    275                 280                 285

        Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile
                    290                 295                 300

  10    Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr
        305             310                 315                 320

        Leu Lys Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe
                    325                 330                 335
  15
        Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His
                    340                 345                 350

        Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile
  20                355                 360                 365

        Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu
            370                 375                 380
```

25   (2) INFORMATION FOR SEQ ID NO:140:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
  30   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

35

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:

40   TGGACCACCC AGAAGTACCT TCATTATGAC   30

        (2) INFORMATION FOR SEQ ID NO:141:

        (i) SEQUENCE CHARACTERISTICS:
  45   (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

50   (ii) MOLECULE TYPE: cDNA

- 219 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:

GTCATAATGA AGGTACTTCT GGGTGGTCCA   30

(2) INFORMATION FOR SEQ ID NO:142:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:

GGACCACCCA GCTTCATTAT GACGAAGAAA C   31

(2) INFORMATION FOR SEQ ID NO:143:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:

GTTTCTTCGT CATAATGAAG CTGGGTGGTC C   31

(2) INFORMATION FOR SEQ ID NO:144:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 29 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

- 220 -

(ii) MOLECULE TYPE: cDNA

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:

GTGGACCACC CAGGACGAAG AAACCTCTC   29

(2) INFORMATION FOR SEQ ID NO:145:
10

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 29 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
15   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

20

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:145:

GAGAGGTTTC TTCGTCCTGG GTGGTCCAC   29
25

(2) INFORMATION FOR SEQ ID NO:146:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 29 base pairs
30   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA
35

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:146:
40
CGTTTCCTCC AAAGTTCCTT CATTATGAC   29

(2) INFORMATION FOR SEQ ID NO:147:

45   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 29 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
50
        (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:147:

GTCATAATGA AGGAACTTTG GAGGAAACG   29

(2) INFORMATION FOR SEQ ID NO:148:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:148:

GGAAACGTTT CCTGCAAAGT ACCTTCATTA TG   32

(2) INFORMATION FOR SEQ ID NO:149:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:149:

CATAATGAAG GTACTTTGCA GGAAACGTTT CC   32

(2) INFORMATION FOR SEQ ID NO:150:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 222 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:150:

5   CACGCAAAAG TCGGGAATAG ATGTCAC   27

(2) INFORMATION FOR SEQ ID NO:151:

     (i) SEQUENCE CHARACTERISTICS:
10   (A) LENGTH: 27 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

15   (ii) MOLECULE TYPE: cDNA

20   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:151:

GTGACATCTA TTCCCGACTT TTGCGTG   27

(2) INFORMATION FOR SEQ ID NO:152:
25

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 25 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
30   (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

35

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:152:

CACCCTGTCG GAAGAGGCCT TCTTC   25
40
(2) INFORMATION FOR SEQ ID NO:153:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 25 base pairs
45   (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA
50

- 223 -

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:153:

GAAGAAGGCC TCTTCCGACA GGGTG   25

   (2) INFORMATION FOR SEQ ID NO:154:
10
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
15   (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: cDNA

20

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:154:

TGACCTCTCG GAAAGCAGCG TGCA   24
25
   (2) INFORMATION FOR SEQ ID NO:155:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
30   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: cDNA
35

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:155:
40
TGCACGCTGC TTTCCGAGAG GTCA   24

   (2) INFORMATION FOR SEQ ID NO:156:

45   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

50
       (ii) MOLECULE TYPE: cDNA

- 224 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:156:

CCTCGAAATC GAGCGAGCAG CTCC   24

(2) INFORMATION FOR SEQ ID NO:157:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:157:

CGATTTCGAG GTCTTTCTCG TTCTC   25

(2) INFORMATION FOR SEQ ID NO:158:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:158:

CCGTGAAAAT AAGCTCGTTA TAACTAGGAA TGG   33

(2) INFORMATION FOR SEQ ID NO:159:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

- 225 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:159:

5   CCATTCCTAG TTATAACGAG CTTATTTTCA CGG   33

(2) INFORMATION FOR SEQ ID NO:160:

     (i) SEQUENCE CHARACTERISTICS:
10   (A) LENGTH: 38 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

15   (ii) MOLECULE TYPE: cDNA

20   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:160:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG   38

(2) INFORMATION FOR SEQ ID NO:161:
25

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 44 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
30   (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

35

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:161:

CCTCTCTCGA GTCAGGTGAC ATCTATTCCA CACTTTTGCG TGGC   44
40
(2) INFORMATION FOR SEQ ID NO:162:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 38 base pairs
45   (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

50

— 226 —

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:162:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG   38

(2) INFORMATION FOR SEQ ID NO:163:
10

            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 38 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
15   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

20

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:163:

CCTCTCTCGA GTCAAGGAAC AGCAAACCTG AAGAAGGC   38
25
(2) INFORMATION FOR SEQ ID NO:164:

            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 38 base pairs
30   (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA
35

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:164:
40
CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG   38

(2) INFORMATION FOR SEQ ID NO:165:

45   (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 38 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear
50
        (ii) MOLECULE TYPE: cDNA

- 227 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:165:

CCTCTCTCGA GTCACTCTGT GGTGAGGTTC GAGTGGCC   38

(2) INFORMATION FOR SEQ ID NO:166:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 38 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:166:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG   38

(2) INFORMATION FOR SEQ ID NO:167:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 38 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:167:

CCTCTCTCGA GTCAGGATGT TTTCAAGTGC TTGAGGGC   38

(2) INFORMATION FOR SEQ ID NO:168:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 16 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

- 228 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:168:

    Met Lys His His His His His His His Ala Ser Val Asn Ala Leu Glu
    1   5   10   15

(Rel no. 12/95 Pub NTS)	FORM 13-27	13-205
Applicant's or agent's file reference number	A-378-CIP2	International application No.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description
on page __15, 16, 45, 60, 64, 67, 100__ line __Too many to list__

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

   American Type Culture Collection (ATCC)

Address of depositary institution (including postal code and country)

   12301 Parklawn Drive
   Rockville, MD 20852

Date of deposit	Accession Number
12/27/95 and 7/24/96	69969, 69970, 69971, 98112 and 98113

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications, e.g., "Accession Number of Deposit")

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer Elnora Rivera Paralegal Specialist IAPD - PCT Operations (703) 305-3678	Authorized officer

Form PCT/RO/134 (July 1992)

(Indications Relating to a Deposited Microorganism (Form PCT/RO/134) [13-27]—page 1 of 1)

- 230 -

WHAT IS CLAIMED IS:

1.   An isolated nucleic acid encoding a
5   polypeptide comprising at least one of the biological
activities of OPG wherein the nucleic acid is selected
from the group consisting of:
a) the nucleic acids shown in Figures 2B-
2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D
10   (SEQ ID NO:124) or complementary strands thereof;
b) nucleic acids which hybridize under
stringent conditions with the polypeptide-encoding
regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B
(SEQ ID NO:122) and 9C-9D (SEQ ID NO:124);
15   c) nucleic acids which hybridize under
stringent conditions with nucleotides 148 through 337
inclusive as shown in Figure 1A; and
d) nucleic acid which are degenerate to
the nucleic acids of (a), (b) and (c).
20

2.   The nucleic acid of Claim 1 which is cDNA,
genomic DNA, synthetic DNA or RNA.

3.   A polypeptide encoded by the nucleic acid
25   of Claim 1.

4.   The nucleic acid of Claim 1 including one
or more codons preferred for Escherichia coli
expression.
30

5.   The nucleic acid of Claim 1 having a
detectable label attached thereto.

6.   The nucleic acid of Claim 1 comprising the
polypeptide-encoding region of Figure 2B-2C (SEQ ID
NO:120), Figure 9A-9B (SEQ ID NO:122) or Figure 9C-9D
(SEQ ID NO:124).

5

7.   The nucleic acid of Claim 6 having the
sequence as shown in Figure 9C-D (SEQ ID NO:124) from
nucleotides 158-1297.

10   8.   An expression vector comprising the
nucleic acid of Claim 1.

9.   The expression vector of Claim 8 wherein
the nucleic acid comprises the polypeptide - encoding
15   region as shown in Figure 9C-9D (SEQ ID NO:124).

10.   A host cell transformed or transfected
with the expression vector of Claim 8.

20   11.   The host cell of Claim 10 which is a
eucaryotic cell.

12.   The host cell of Claim 11 which is
selected from the group consisting of CHO, COS, 293,
25   3T3, CV-1 and BHK cells.

13.   The host cell of Claim 10 which is a
procaryotic cell.

30   14. The host cell of Claim 13 which is
Escherichia coli.

15.   A transgenic mammal comprising the
expression vector of Claim 8.

16.   The transgenic mammal of Claim 15 which
is a rodent.

5   17.   The transgenic mammal of Claim 16 which
is a mouse.

18.   A process for the production of OPG
comprising:
10   growing under suitable nutrient
conditions host cells transformed or transfected with
the nucleic acid of Claim 1; and
                isolating the polypeptide products of the
expression of the nucleic acids.
15

19.   A purifed and isolated polypeptide
comprising OPG.

20.   The polypeptide of Claim 19 which is
20   mammalian OPG.

21.   The polypeptide of Claim 20 which is human
OPG.

25   22.   The polypeptide of Claim 19 which is
substantially free of other human proteins.

23.   The polypeptide of Claim 21 having the
amino acid sequence as shown in Figure 2B-2C (SEQ ID
30   NO:121), Figure 9A-9B (SEQ ID NO:123), or Figure 9C-9D
(SEQ ID NO:125) or a derivative thereof.

24.   The polypeptide of Claim 23 having the
amino acid sequence as shown in Figure 9C-9D (SEQ ID
NO:125) from residues 22-401 inclusive.

5   25. The polypeptide of Claim 23 having the
amino acid sequence as shown in Figure 9C-9D (SEQ ID
NO:125) from residues 32-401 inclusive.

26.   The polypeptide of Claim 19 which is
10   characterized by being a product of expression of an
exogenous DNA sequence.

27.   The polypeptide of Claim 26 wherein the
DNA is cDNA, genomic DNA or synthetic DNA.

15

28.   The polypeptide of Claim 19 which has
been modified with a water-soluble polymer.

29.   The polypeptide of Claim 28 wherein the
20   water soluble polymer is polyethylene glycol.

30.   A polypeptide comprising:
an amino acid sequence of at least about 164
amino acids comprising four cysteine-rich domains
25   characteristic of the cysteine rich domains of tumor
necrosis factor receptor extracellular regions; and
an activity of increasing bone density.

31.   A polypeptide comprising the amino acid
30   sequence as shown in Figure 2B-2C (SEQ ID NO:121),
Figure 9A-9B (SEQ ID NO:123) or Figure 9C-9D (SEQ ID
NO:125) having an amino terminus at residue 22, and
wherein from 1 to 216 amino acids are deleted from the
carboxy terminus.

32.   The polypeptide of Claim 31 comprising
the amino acid sequence from residues 22-185, 22-189,
22-194, or 22-201 inclusive.

5

33.   The polypeptide of Claim 32 further
comprising an Fc region of human IgG1 extending from the
carboxy terminus.

10   34.   A polypeptide comprising the amino acid
sequence as shown in Figure 2B-2C (SEQ ID NO:121),
Figure 9A-9B (SEQ ID NO:123) or Figure 9C-9D (SEQ ID
NO:125) having an amino terminus at residue 22, wherein
from 1 to 10 amino acids are deleted from the amino
15   terminus and, optionally, from 1 to 216 amino acids are
deleted from the carboxy terminus.

35.   The polypeptide of Claim 34 comprising
the amino acid sequence from residues 27-185, 27-189,
20   27-194, 27-401, or 32-401 inclusive.

36.   The polypeptide of Claim 35 further
comprising an Fc region of human IgG1 extending from the
carboxy terminus.

25

37.   A polypeptide selected from the group
consisting of:
          huOPG [22-201]-Fc
          huOPG [22-401]-Fc
30   huOPG [22-180]-Fc
          huOPG met [22-401]-Fc
          huOPG Fc-met [22-401]
          huOPG met [22-185]
          huOPG met [22-189]

<pre>
        huOPG met [22-194]
        huOPG met [27-185]
        huOPG met [27-189]
        huOPG met [27-194]
  5   huOPG met [32-401]
        huOPG met-lys[22-401]
        huOPG met [22-401]
        huOPG met [22-401]-Fc (P25A)
        huOPG met [22-401] (P25A)
 10   huOPG met [22-401] (P26A)
        huOPG met [22-401] (P26D)
        huOPG met [22-194] (P25A)
        huOPG met [22-194] (P26A)
        huOPG met met-(lys)$_3$ [22-401]
 15   huOPG met met-arg-gly-ser-(his)$_6$ [22-401]
</pre>

38.   A nucleic acid encoding the polypeptide of Claim 37.

20   39.   An antibody or fragment thereof which specifically binds to OPG.

40.   The antibody of Claim 39 which is a monoclonal antibody.

25

41.   A method for detecting the presence of OPG in a biological sample comprising:
        incubating the sample with the antibody of Claim 39 under conditions that allow binding of the
30 antibody to OPG; and
        detecting the bound antibody.

- 236 -

42. A method to assess the ability of a candidate substance to bind to OPG comprising:
                incubating OPG with the candidate substance
5   under conditions that allow binding; and
                measuring the bound substance.

43. A method of regulating the levels of OPG in an animal comprising modifying the animal with a
10 nucleic acid encoding OPG.

44. The method of Claim 43 wherein the nucleic acid promotes an increase in the tissue level of OPG.
15

45. The method of Claim 44 wherein the animal is a human.

46. A pharmaceutical composition comprising a
20 therapeutically effective amount of OPG in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.

47. The composition of Claim 46 wherein the
25 OPG is human OPG.

48. The composition of Claim 47 wherein the OPG has the amino acid sequence as shown in Figure 9B.

30   49. A method of treating a bone disorder comprising administering a therapeutically effective amount of the polypeptide of Claim 19.

- 237 -

50. The method of Claim 49 wherein the polypeptide is human OPG.

51. The method of Claim 49 wherein the bone
5   disorder is excessive bone loss.

52. The method of Claim 51 wherein the bone
disorder is selected from the group consisting of
osteoporosis, Paget's disease of bone, hypercalcemia,
10   hyperparathyroidism, steroid-induced osteopenia, bone
loss due to rheumatoid arthritis, bone loss due to
osteomyelitis, osteolytic metastasis, and periodontal
bone loss.

15   53. The method of Claim 49 further comprising
administering a therapeutically effective amount of a
substances selected from the group consisting of bone
morphogenic proteins BMP-1 through BMP-12, TGF-β family
members, IL-1 inhibitors, TNFα inhibitors, parathyroid
20   hormone and analogs thereof, parathyroid hormone related
protein and analogs thereof, E series prostaglandins,
bisphosphonates, and bone-enhancing minerals.

54. An osteoprotegerin multimer consisting of
25   osteoprotegerin monomers.

55. The multimer of Claim 54 which is a
dimer.

30   56. The multimer of Claim 54 formed by
interchain disulfide bonds.

57. The multimer of Claim 54 formed by
association Fc regions derived from human IgG1.

- 238 -

58.   The multimer of Claim 54 which is
essentially free of osteoprotegerin monomers and
inactive multimers.

5

59.   The multimer of Claim 54 wherein the
monomers comprise the amino acid sequence as shown in
Figure 9C-9D (SEQ ID NO:125) from residues 22-401, or a
derivative thereof.

10

60.   The multimer of Claim 54 wherein the
monomers comprise the amino acid sequence as shown in
Figure 9C-9D (SEQ ID NO:125) from residues 22-194.

FIG.1A

```
              148       178       208       238       268       298
FRI-1         ALLVFLDIIEWTTQETFPPKYLHYDPETGRQLLCDKCAPGTYLKQHCTVRRKTLCVPCPD
                                                   : |:||:|| | || |:| :|| |
SW:TNR2_HUMAN HALPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQHAKVFCTKTSDTVCDSCED
                   30        40        50        60        70        80

              328
FRI-1         YSYTDSWHTS
                :|: |:
SW:TNR2_HUMAN STYTQLMNWVPECLSCGSRCSSDQVETQACTREQNRICTCRPGWYCALSKQEGCRLCAPL
                   90        100       110       120       130       140
```

FIG.1B

```
FRI-1         69  YLHYDPETGRQLLCDKCAPGTYLKQHC.TVRRKTLCV.PCPDY.SYTDSW
                  | : ... :| ||:| .| .: | | :| : |.|. .
TNFR profile  6   YHYYDQNGRMCEECHMCQPGHFLVKHCKQPKRDTVCHKPCEPGVTYTDDW

FRI-1         116 H
                  |
TNFR profile  56  H
```

Z Score = 8.29

FIG. IC
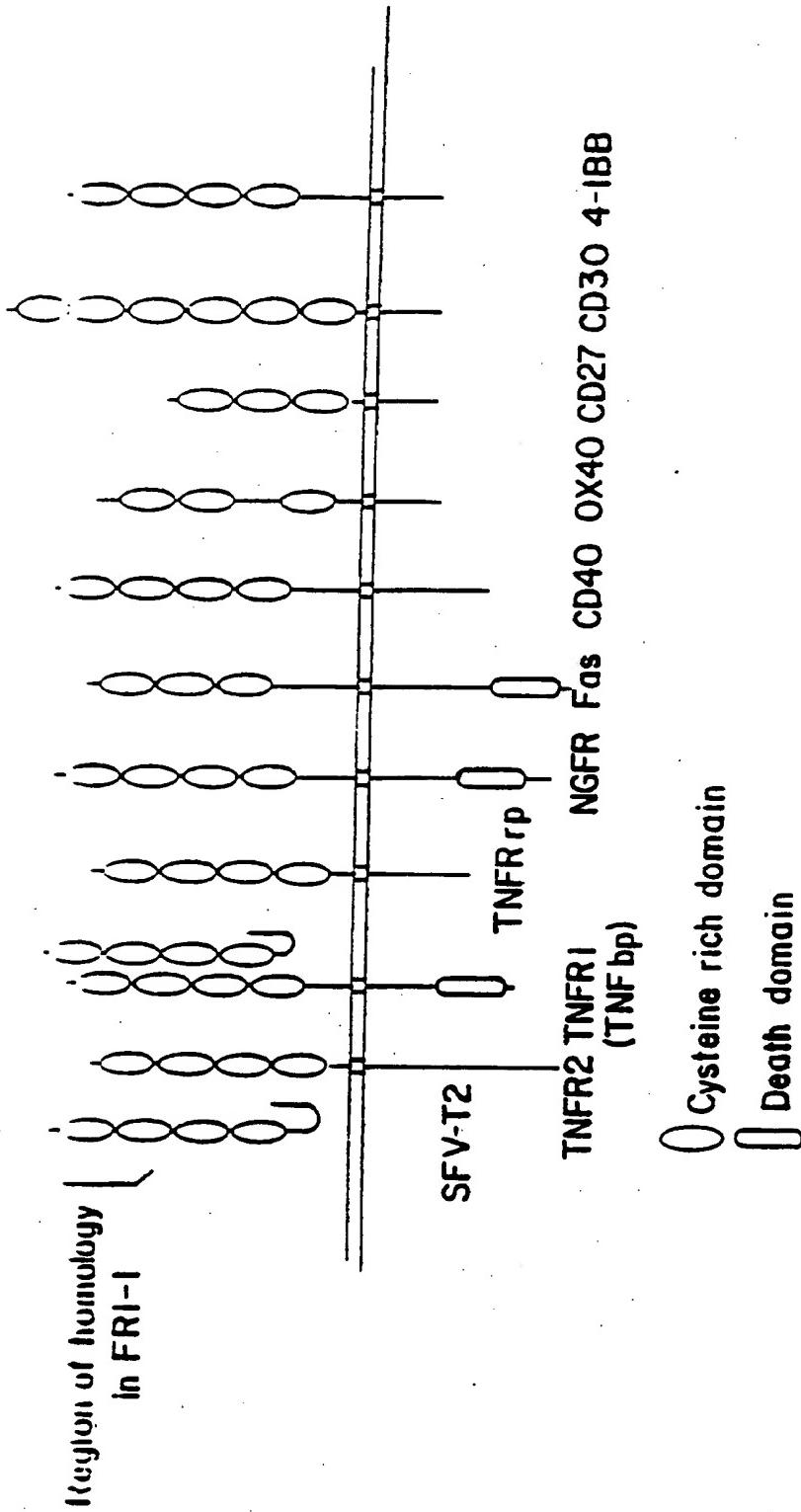
FIG.2A

FIG.2B

```
            10                    30                    50
ATCAAAGGCAGGGCATACTTCCTGTTGCCCAGACCTTATATAAAACGTCATGTTCGCCTG
            70                    90                    110
GGCAGCAGAGAAGCACCTAGCACTGGCCCAGCGGCTGCCGCCTGAGGTTTCCAGAGGACC
            130                   150                   170
ACAATGAACAAGTGGCTGTGCTGTGCACTCCTGGTGTTCTTGGACATCATTGAATGGACA
             M  N  K  W  L  C  C  A  L  L  V  F  L  D  I  I  E  W  T
            190                   210                   230
ACCCAGGAAACCTTTCCTCCAAAATACTTGCATTATGACCCAGAAACCGGACGTCAGCTC
             T  Q  E  T  F  P  P  K  Y  L  H  Y  D  P  E  T  G  R  Q  L
            250                   270                   290
TTGTGTGACAAATGTGCTCCTGGCACCTACCTAAAACAGCACTGCACAGTCAGGAGGAAG
             L  C  D  K  C  A  P  G  T  Y  L  K  Q  H  C  T  V  R  R  K
            310                   330                   350
ACACTGTGTGTCCCTTGCCCTGACTACTCTTATACAGACAGCTGGCACACGAGTGATGAA
             T  L  C  V  P  C  P  D  Y  S  Y  T  D  S  W  H  T  S  D  E
            370                   390                   410
TGCGTGTACTGCAGCCCCGTGTGCAAGGAACTGCAGACCGTGAAACAGGAGTGCAACCGC
             C  V  Y  C  S  P  V  C  K  E  L  Q  T  V  K  Q  E  C  N  R
            430                   450                   470
ACCCACAACCGAGTGTGCGAATGTGAGGAAGGGCGCTACCTGGAGCTCGAATTCTGCTTG
             T  H  N  R  V  C  E  C  E  E  G  R  Y  L  E  L  E  F  C  L
            490                   510                   530
AAGCACCGGAGCTGTCCCCCAGGCTTGGGTGTGCTGCAGGCTGGGACCCCAGAGCGAAAC
             K  H  R  S  C  P  P  G  L  G  V  L  Q  A  G  T  P  E  R  N
            550                   570                   590
ACGGTTTGCAAAAGATGTCCGGATGGGTTCTTCTCAGGTGAGACGTCATCGAAAGCACCC
             T  V  C  K  R  C  P  D  G  F  F  S  G  E  T  S  S  K  A  P
            610                   630                   650
TGTAGGAAACACACCAACTGCAGCTCACTTGGCCTCCTGCTAATTCAGAAAGGAAATGCA
             C  R  K  H  T  N  C  S  S  L  G  L  L  L  I  Q  K  G  N  A
            670                   690                   710
ACACATGACAATGTATGTTCCGGAAACAGAGAAGCAACTCAAAATTGTGGAATAGATGTC
             T  H  D  N  V  C  S  G  N  R  E  A  T  Q  N  C  G  I  D  V
            730                   750                   770
ACCCTGTGCGAAGAGGCATTCTTCAGGTTTGCTGTGCCTACCAAGATTATACCGAATTGG
             T  L  C  E  E  A  F  F  R  F  A  V  P  T  K  I  I  P  N  W
            790                   810                   830
CTGAGTGTTCTGGTGGACAGTTTGCCTGGGACCAAAGTGAATGCAGAGAGTGTAGAGAGG
             L  S  V  L  V  D  S  L  P  G  T  K  V  N  A  E  S  V  E  R
            850                   870                   890
ATAAAACGGAGACACAGCTCGCAAGAGCAAACTTTCCAGCTACTTAAGCTGTGGAAGCAT
             I  K  R  R  H  S  S  Q  E  Q  T  F  Q  L  L  K  L  W  K  H
            910                   930                   950
CAAAACAGAGACCAGGAAATGGTGAAGAAGATCATCCAAGACATTGACCTCTGTGAAAGC
             Q  N  R  D  Q  E  M  V  K  K  I  I  Q  D  I  D  L  C  E  S
            970                   990                   1010
AGTGTGCAACGGCATATCGGCCACGCGAACCTCACCACAGAGCAGCTCCGCATCTTGATG
             S  V  Q  R  H  I  G  H  A  N  L  T  T  E  Q  L  R  I  L  M
```

FIG.2C

```
         1030               1050                    1070
GAGAGCTTGCCTGGGAAGAAGATCAGCCCAGACGAGATTGAGAGAACGAGAAAGACCTGC
 E    L   P   G   K   K   I   S   P   D   E   I   E   R   T   R   K   T   C
         1090               1110                    1130
AAACCCAGCGAGCAGCTCCTGAAGCTACTGAGCTTGTGGAGGATCAAAAATGGAGACCAA
 K    P   S   E   Q   L   L   K   L   L   S   L   W   R   I   K   N   G   D   Q
         1150               1170                    1190
GACACCTTGAAGGGCCTGATGTACGCACTCAAGCACTTGAAAGCATACCACTTTCCCAAA
 D    T   L   K   G   L   M   Y   A   L   K   H   L   K   A   Y   H   F   P   K
         1210               1230                    1250
ACCGTCACCCACAGTCTGAGGAAGACCATCAGGTTCTTGCACAGCTTCACCATGTACCGA
 T    V   T   H   S   L   R   K   T   I   R   F   L   H   S   F   T   M   Y   R
         1270               1290                    1310
TTGTATCAGAAACTCTTTCTAGAAATGATAGGGAATCAGGTTCAATCAGTGAAGATAAGC
 L    Y   Q   K   L   F   L   E   M   I   G   N   Q   V   Q   S   V   K   I   S
         1330               1350                    1370
TGCTTATAGTTAGGAATGGTCACTGGGCTGTTTCTTCAGGATGGGCCAACACTGATGGAG
 C    L
         1390               1410                    1430
CAGATGGCTGCTTCTCCGGCTCTTGAAATGGCAGTTGATTCCTTTCTCATCAGTTGGTGG
         1450               1470                    1490
GAATGAAGATCCTCCAGCCCAACACACACACTGGGGAGTCTGAGTCAGGAGAGTGAGGCA
         1510               1530                    1550
GGCTATTTGATAATTGTGCAAAGCTGCCAGGTGTACACCTAGAAAGTCAAGCACCCTGAG
         1570               1590                    1610
AAAGAGGATATTTTTATAACCTCAAACATAGGCCCTTTCCTTCCTCTCCTTATGGATGAG
         1630               1650                    1670
TACTCAGAAGGCTTCTACTATCTTCTGTGTCATCCCTAGATGAAGGCCTCTTTTATTTAT
         1690               1710                    1730
TTTTTTATTCTTTTTTTCGGAGCTGGGGACCGAACCCAGGGCCTTGCGCTTGCGAGGCAA
         1750               1770                    1790
GTGCTCTACCACTGAGCTAAATCTCCAACCCCTGAAGGCCTCTTTCTTTCTGCCTCTGAT
         1810               1830                    1850
AGTCTATGACATTCTTTTTTCTACAATTCGTATCAGGTGCACGAGCCTTATCCCATTTGT
         1870               1890                    1910
AGGTTTCTAGGCAAGTTGACCGTTAGCTATTTTTCCCTCTGAAGATTTGATTCGAGTTGC
         1930               1950                    1970
AGACTTGGCTAGACAAGCAGGGGTAGGTTATGGTAGTTTATTTAACAGACTGCCACCAGG
         1990               2010                    2030
AGTCCAGTGTTTCTTGTTCCTCTGTAGTTGTACCTAAGCTGACTCCAAGTACATTTAGTA
         2050               2070                    2090
TGAAAAATAATCAACAAATTTTATTCCTTCTATCAACATTGGCTAGCTTTGTTTCAGGGC
         2110               2130                    2150
ACTAAAAGAAACTACTATATGGAGAAAGAATTGATATTGCCCCCAACGTTCAACAACCCA
         2170               2190                    2210
ATAGTTTATCCAGCTGTCATGCCTGGTTCAGTGTCTACTGACTATGCGCCCTCTTATTAC
         2230               2250                    2270
TGCATGCAGTAATTCAACTGGAAATAGTAATAATAATAGAAATAAAATCTAGACTCC
         2290               2310                    2330
ATTGGATCTCTCTGAATATGGGAATATCTAACTTAAGAAGCTTTGAGATTTCAGTTGTGT
         2350               2370                    2390
TAAAGGCTTTTATTAAAAAGCTGATGCTCTTCTGTAAAAGTTACTAATATATCTGTAAGA
         2410               2430
CTATTACAGTATTGCTATTTATATCCATCCAG
```

FIG. 2D

Multiple sequence alignment (sequences shown rotated 90° in the original figure).

Block 1 — rows and alignment end positions:

Sequence	Position
fas.frg	45
tnfr1.frg	44
sfv-t2.frg	25
tnfr2.frg	39
cd40.frg	28
osteo.frg	26
ngfr.frg	34
ox40.frg	28
41bb.frg	25

Block 2 — rows and alignment end positions:

Sequence	Position
fas.frg	95
tnfr1.frg	94
sfv-t2.frg	74
tnfr2.frg	86
cd40.frg	72
osteo.frg	75
ngfr.frg	78
ox40.frg	72
41bb.frg	54

Block 3 — rows and alignment end positions:

Sequence	Position
fas.frg	103
tnfr1.frg	144
sfv-t2.frg	84
tnfr2.frg	98
cd40.frg	85
osteo.frg	65
ngfr.frg	89
ox40.frg	84
41bb.frg	65

FIG.2E

Block 1:

fas.frg	- C R L C D E G H G L E V E I N C T R T Q N T K C R C K P N F F C N S T V C E H C D P C T K C E H G	152
tnfr1.frg	N C S L C L N G - - T V H L S C Q E K Q N T V C T C H A G F F L R E N E C V S C S N C K K S L E C	191
sfv-t2.frg	- - - - P C T G H L S E S Q P C D R T H D R V C N C C S T G N Y C A L S K Q E G Q R I C C A P Q T K C	129
tnfr2.frg	- - - - R C S S D V E T Q A C T R E Q N R I C T C R P G W Y C A L S K D C E C C R L C C A P L R K C C	143
cd40.frg	- - - - N Q G L R V K K E G T A E S D T V C T C K E G Q H C T - - A C C A Q H T P C	125
osteo.frg	- - - - V C K E L T V K Q E C N R T H N R V C E C G R Y - - L E L E - F C L K H R S C	124
ngfr.frg	- - - - V G L Q S M I - S A P C D D A V C C R C A Y G Y Y - Q D E T T G R C	128
ox40.frg	- - - - R S G S E L - K Q N C C T P T E D T C - R P G T Q P R Q D S S H - -	116
41bb.frg	- - - - V C A G Y E R F K K F C S S T H N A E C I E G F H C L G P Q C T R C - - E K D C	105

Block 2:

fas.frg	- - - - I K E C T L T S N T K C K E - E G S R S N L - G W L C L L P I P L I	187
tnfr1.frg	- - - - T K L C L P Q I E N V K G T E - D S G T T V L L P C L L S L L F I	230
sfv-t2.frg	P A G Y G V S - C H T R A G D T L C E K P C A P G T E T S D V I E F G L C L S V G F N L Y	178
tnfr2.frg	R P G F G V A R P G T E T S D V V C K C R P G T F C Y I S N V V A I P G N	193
cd40.frg	I P G F G V M E M A T E T T D T V C H P C P V G F F S N Q E T S C E D K N L E V L	175
osteo.frg	P P G L G V L Q A G T P E R N T V C K R C P D D G T Y S D G L L I	174
ngfr.frg	- - E A G S G L V F S C Q D K Q N T V C E E C P P G H E S P G S N - T E R Q L R	178
ox40.frg	- - - - - - K L G V D C V P C P P G H E S P G S N - Q A K P W T N C L S G K Q I R	152
41bb.frg	R P G Q E L T K Q G - - - - C K T C S L G T F N D Q - N G T G V C R P W T N C S L D G R S V L	147

Block 3:

fas.frg	V W V - - - - - - K R K E V Q K T C R K H R K E N Q G S H E S P T L N P E - - - - - - - T	219
tnfr1.frg	G L M Y R Y Q R W K S K L Y S I V C G K S T P E K E G E L E G T T T A - - - - - S P T P G F T	280
sfv-t2.frg	P V N - - - - - - E T S C T T T A - G H N E V I K T K E E T V T L N Y T	207
tnfr2.frg	A S R - - - - - - D A V C C T S P T R S H A P G A V H L P Q P V S T R S Q H T	227
cd40.frg	Q K G - - - - T S Q T N A T H D N - I C G G L K S R M R - A L V	197
osteo.frg	Q K G - - - - N R E A T Q N C G I D V T L C E E A F F R	208
ngfr.frg	E C T R W A D A E C E E I P G R - W I T R S T - P P E G S D S T A P S T Q E P E A P P E Q D L R	224
ox40.frg	H P A S N S V C E D R S L L A T L L W E T Q R T T F E R P T T V P R T S Q L P S T P T L V	202
41bb.frg	K T G T T E K D V V C G P P V V S F S P S T T I S V T P E G G P G - - - G H S L Q V L T L F L	191
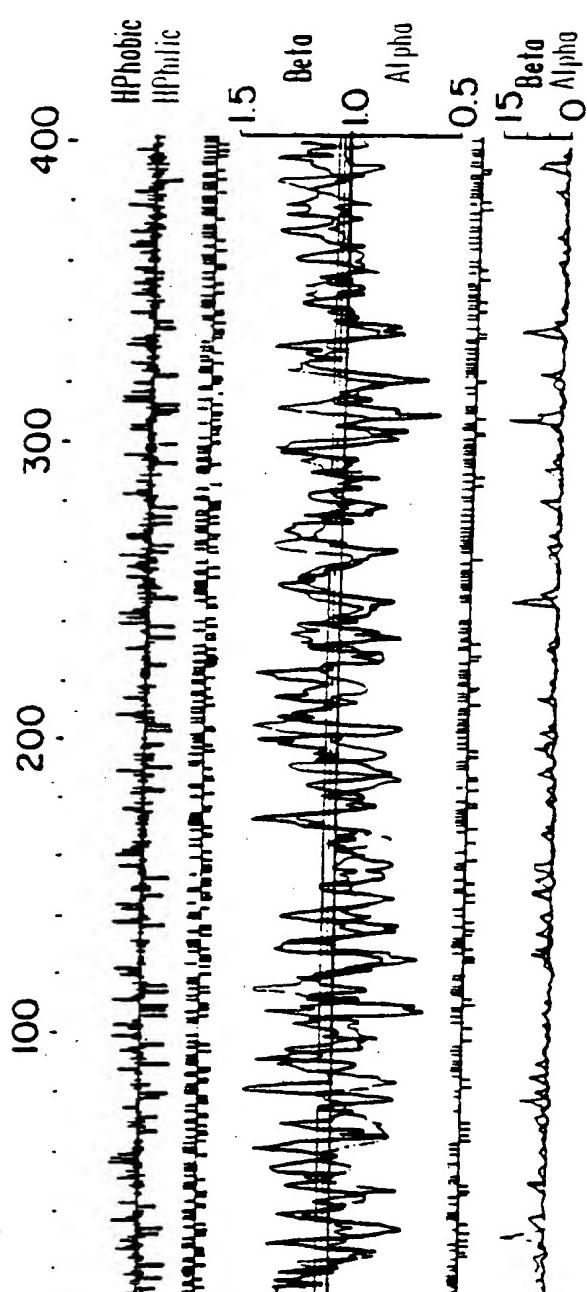
FIG.3A

FIG.3B

FIG.3C

FIG.3D

FIG.3E

FIG.3F COOH End

FIG.3G Turn

FIG.3H Hydrophobic Moment

FIG.3 I Goldman et al Kyte-Doolittle
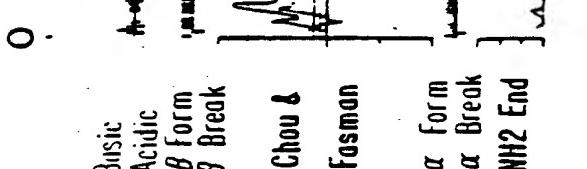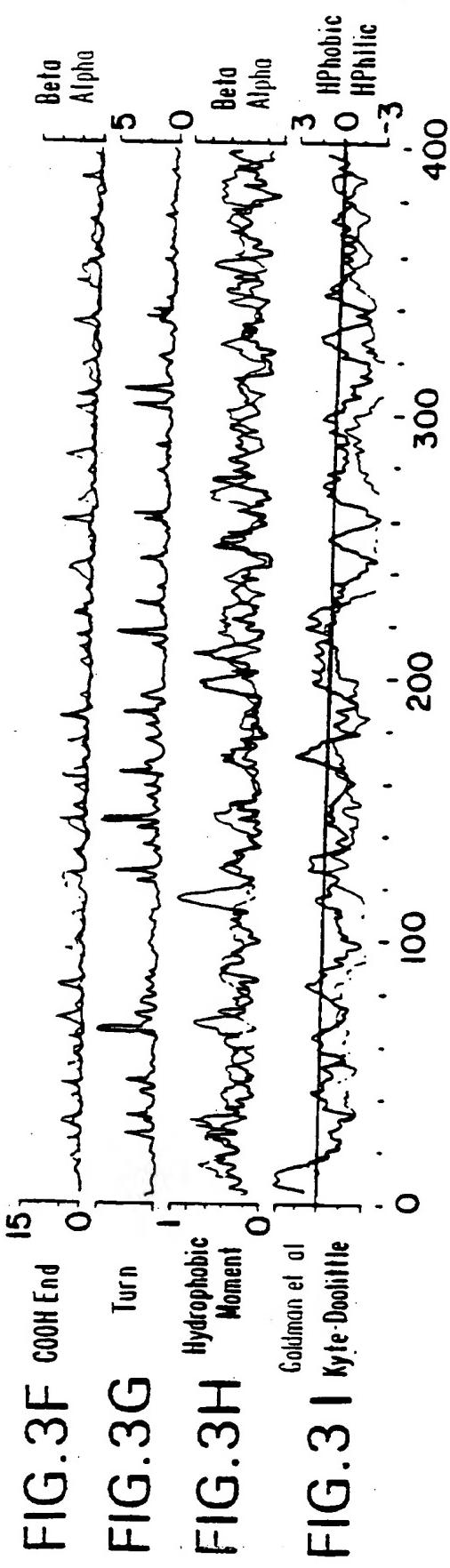
FIG.5
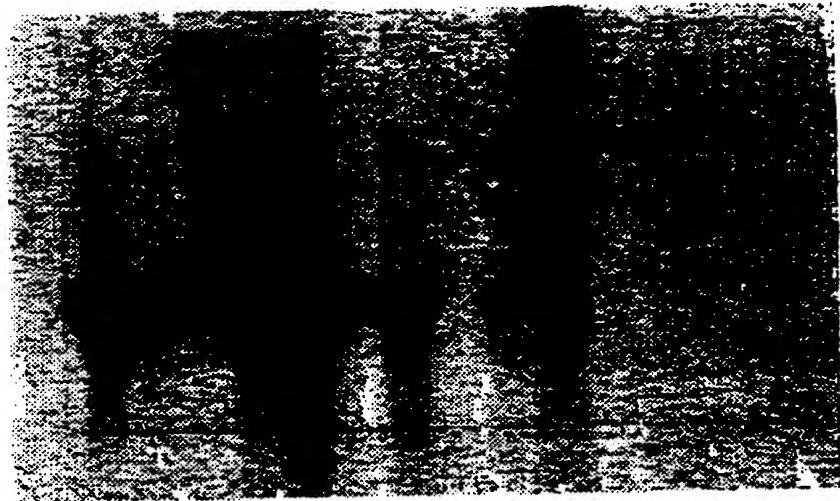
2 11 16 17 22 28 33 38 45 Kb 1 12 18 30

Transgenic Founders   **Controls**

FIG.4A　　　　FIG.4B

Human Immune System

Adult Human　　　　Fetal Human

Heart Brain Placenta Lung Liver Sk. Muscle Kidney Pancreas Brain Lung Liver Kidney

Fetal liver Bone Marrow PBL's Appendix Thymus Lymph node Spleen

9.5 ►
7.5 ►
4.5 ►
2.4 ►
1.35►
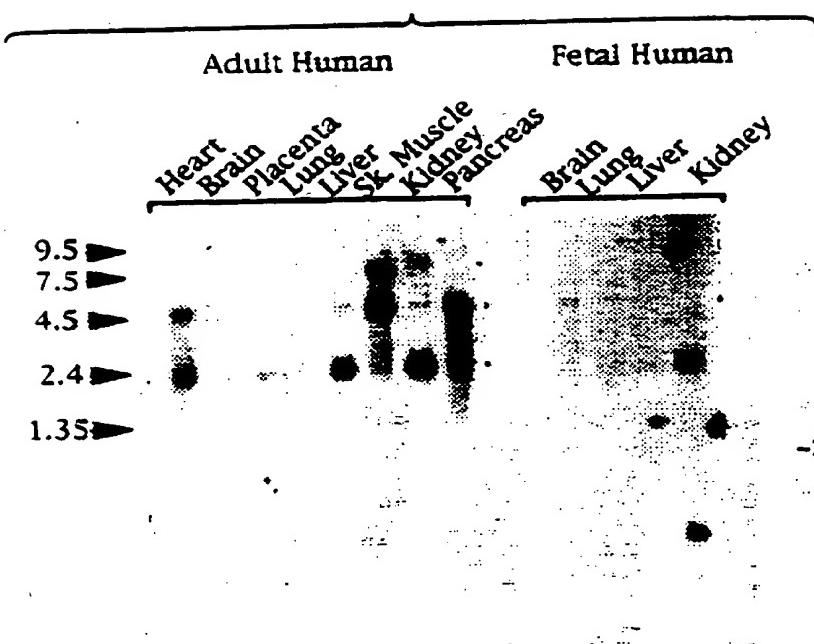
-2.4 kb ►
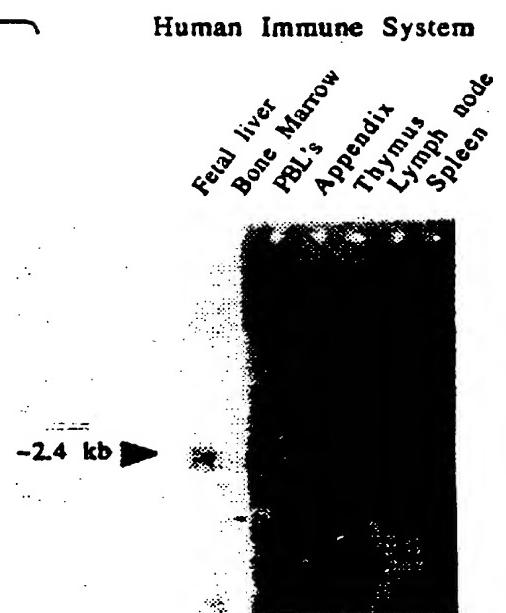
FIG.6A
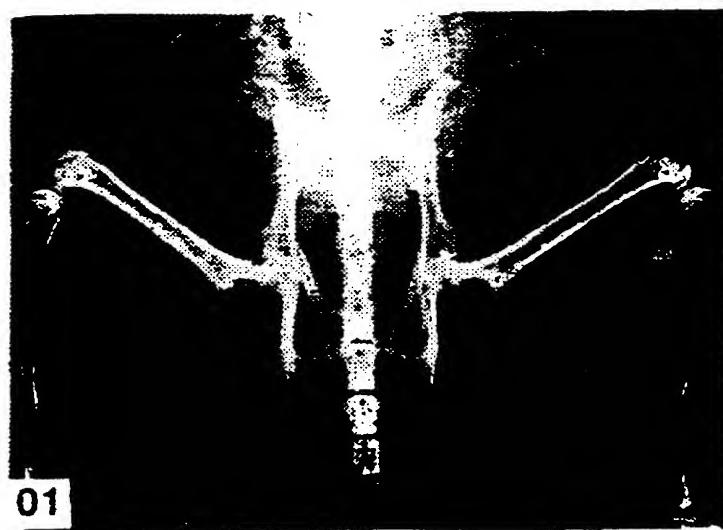
FIG.6B
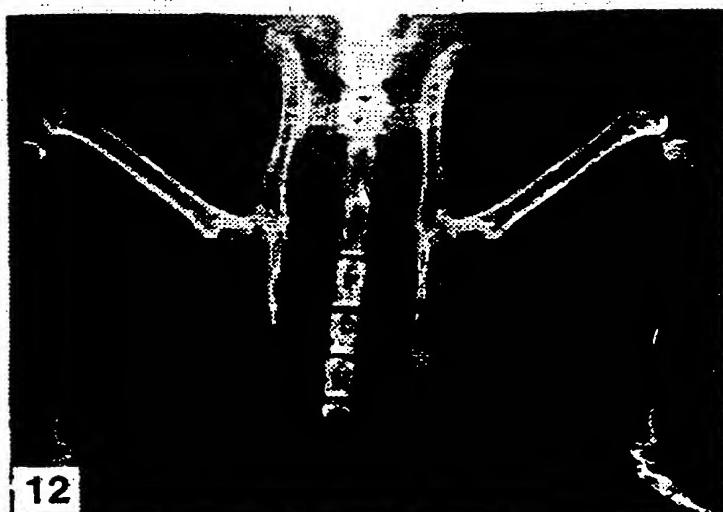
FIG.6C
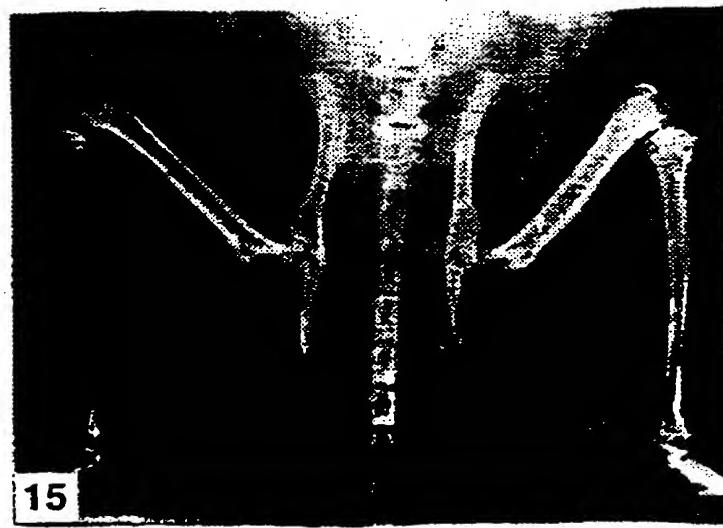
FIG.6D
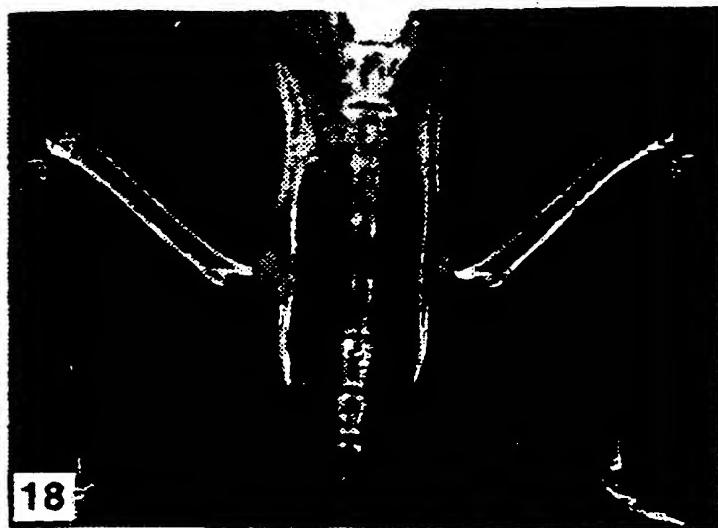
FIG.6E

FIG.6F
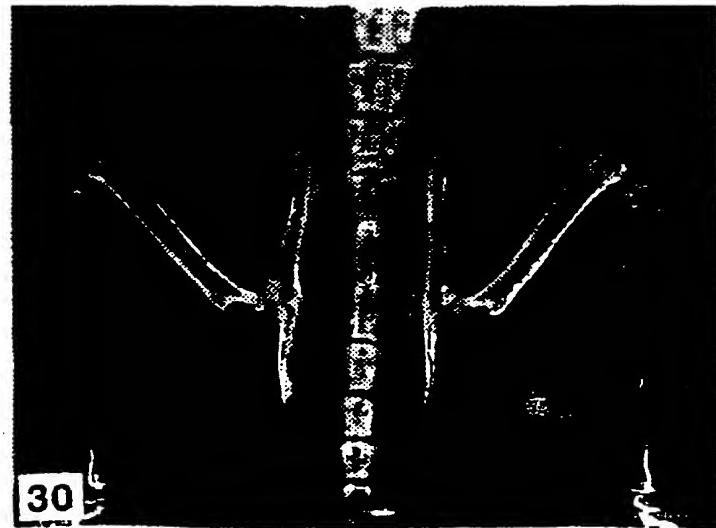
FIG.6G

FIG.6H

FIG.9A

```
        10                      30                         50
CCTTATATAARACGTCATGATTGCCTGGGCTGCAGAGACGCACCTAGCACTGACCCAGCG
        70                      90                        110
GCTGCCTCCTGAGGTTTCCCGAGGACCACAATGAACAAGTGGCTGTGCTGCGCACTCCTG
                                         M  N  K  W  L  C  C  A  L  L
        130                     150                        170
GTGCTCCTGGACATCATTGAATGGACAACCCAGGAAACCCTTCCTCCAAAGTACTTGCAT
 V  L  L  D  I  I  E  W  T  T  Q  E  T  L  P  P  K  Y  L  H
        190                     210                        230
TATGACCCAGAAACTGGTCATCAGCTCCTGTGTGACAAATGTGCTCCTGGCACCTACCTA
 Y  D  P  E  T  G  H  Q  L  L  C  D  K  C  A  P  G  T  Y  L
        250                     270                        290
AAACAGCACTGCACAGTGAGGAGGAAGACATTGTGTGTCCCTTGCCCTGACCACTCTTAT
 K  Q  H  C  T  V  R  R  K  T  L  C  V  P  C  P  D  H  S  Y
        310                     330                        350
ACGGACAGCTGGCACACCAGTGATGAGTGTGTGTATTGCAGCCCAGTGTGCAAGGAACTG
 T  D  S  W  H  T  S  D  E  C  V  Y  C  S  P  V  C  K  E  L
        370                     390                        410
CAGTCCGTGAAGCAGGAGTGCAACCGCACCCACAACCGAGTGTGTGAGTGTGAGGAAGGG
 Q  S  V  K  Q  E  C  N  R  T  H  N  R  V  C  E  C  E  E  G
        430                     450                        470
CGTTACCTGGAGATCGAATTCTGCTTGAAGCACCGGAGCTGTCCCCCGGGCTCCGGCGTG
 R  Y  L  E  I  E  F  C  L  K  H  R  S  C  P  P  G  S  G  V
        490                     510                        530
GTGCAAGCTGGAACCCCAGAGCGAAACACAGTTTGCAAAAAATGTCCAGATGGGTTCTTC
 V  Q  A  G  T  P  E  R  N  T  V  C  K  K  C  P  D  G  F  F
        550                     570                        590
TCAGGTGAGACTTCATCGAAAGCACCCTGTATAAAACACACGAACTGCAGCACATTTGGC
 S  G  E  T  S  S  K  A  P  C  I  K  H  T  N  C  S  T  F  G
        610                     630                        650
CTCCTGCTAATTCAGAAAGGAAATGCAACACATGACAACGTGTGTTCCGGAAACAGAGAA
 L  L  L  I  Q  K  G  N  A  T  H  D  N  V  C  S  G  N  R  E
        670                     690                        710
GCCACGCAAAAGTGTGGAATAGATGTCACCCTGTGTGAAGAGGCCTTCTTCAGGTTTGCT
 A  T  Q  K  C  G  I  D  V  T  L  C  E  E  A  F  F  R  F  A
        730                     750                        770
GTTCCTACCAAGATTATACCAAATTGGCTGAGTGTTTTGGTGGACAGTTTGCCTGGGACC
 V  P  T  K  I  I  P  N  W  L  S  V  L  V  D  S  L  P  G  T
```

FIG.9B

```
          790                     810                     830
AAAGTGAATGCCGAGAGTGTAGAGAGGATAAAACGGAGACACAGCTCACAAGAGCAAACC
 K  V  N  A  E  S  V  E  R  I  K  R  R  H  S  S  Q  E  Q  T
          850                     870                     890
TTCCAGCTGCTGAAGCTGTGGAAACATCAAAACAGAGACCAGGAAATGGTGAAGAAGATC
 F  Q  L  L  K  L  W  K  H  Q  N  R  D  Q  E  M  V  K  K  I
          910                     930                     950
ATCCAAGACATTGACCTCTGTGAAAGCAGCGTGCAGCGGCATCTCGGCCACTCGAACCTC
 I  Q  D  I  D  L  C  E  S  S  V  Q  R  H  L  G  H  S  N  L
          970                     990                     1010
ACCACAGAGCAGCTTCTTGCCTTGATGGAGAGCCTGCCTGGGAAGAAGATCAGCCCAGAA
 T  T  E  Q  L  L  A  L  M  E  S  L  P  G  K  K  I  S  P  E
          1030                    1050                    1070
GAGATTGAGAGAACGAGAAAGACCTGCAAATCGAGCGAGCAGCTCCTGAAGCTACTCAGT
 E  I  E  R  T  R  K  T  C  K  S  S  E  Q  L  L  K  L  L  S
          1090                    1110                    1130
TTATGGAGGATCAAAAATGGTGACCAAGACACCTTGAAGGGCCTGATGTATGCCCTCAAG
 L  W  R  I  K  N  G  D  Q  D  T  L  K  G  L  M  Y  A  L  K
          1150                    1170                    1190
CACTTGAAAACATCCCACTTTCCCAAAACTGTCACCCACAGTCTGAGGAAGACCATGAGG
 H  L  K  T  S  H  F  P  K  T  V  T  H  S  L  R  K  T  M  R
          1210                    1230                    1250
TTCCTGCACAGCTTCACAATGTACAGACTGTATCAGAAGCTCTTTTTAGAAATGATAGGG
 F  L  H  S  F  T  M  Y  R  L  Y  Q  K  L  F  L  E  M  I  G
          1270                    1290                    1310
AATCAGGTTCAATCCGTGAAAATAAGCTGCTTATAACTAGGAATGGTCACTGGGCTGTTT
 N  Q  V  Q  S  V  K  I  S  C  L

CTTCA
```

FIG.9C

```
          10                         30                         50
GTATATATAACGTGATGAGCGTACGGGTGCGGAGACGCACCGGAGCGCTCGCCCAGCCGC
          70                         90                        110
CGYCTCCAAGCCCCTGAGGTTTCCGGGGACCACAATGAACAAGTTGCTGTGCTGCGCGCT
                                                   M  N  K  L  L  C  C  A  L
          130                        150                       170
CGTGTTTCTGGACATCTCCATTAAGTGGACCACCCAGGAAACGTTTCCTCCAAAGTACCT
 V  F  L  D  I  S  I  K  W  T  T  Q  E  T  F  P  P  K  Y  L
          190                        210                       230
TCATTATGACGAAGAAACCTCTCATCAGCTGTTGTGTGACAAATGTCCTCCTGGTACCTA
 H  Y  D  E  E  T  S  H  Q  L  L  C  D  K  C  P  P  G  T  Y
          250                        270                       290
CCTAAAACAACACTGTACAGCAAAGTGGAAGACCGTGTGCGCCCCTTGCCCTGACCACTA
 L  K  Q  H  C  T  A  K  W  K  T  V  C  A  P  C  P  D  H  Y
          310                        330                       350
CTACACAGACAGCTGGCACACCAGTGACGAGTGTCTATACTGCAGCCCCGTGTGCAAGGA
 Y  T  D  S  W  H  T  S  D  E  C  L  Y  C  S  P  V  C  K  E
          370                        390                       410
GCTGCAGTACGTCAAGCAGGAGTGCAATCGCACCCACAACCGCGTGTGCGAATGCAAGGA
 L  Q  Y  V  K  Q  E  C  N  R  T  H  N  R  V  C  E  C  K  E
          430                        450                       470
AGGGCGCTACCTTGAGATAGAGTTCTGCTTGAAACATAGGAGCTGCCCTCCTGGATTTGG
 G  R  Y  L  E  I  E  F  C  L  K  H  R  S  C  P  P  G  F  G
          490                        510                       530
AGTGGTGCAAGCTGGAACCCCAGAGCGAAATACAGTTTGCAAAAGATGTCCAGATGGGTT
 V  V  Q  A  G  T  P  E  R  N  T  V  C  K  R  C  P  D  G  F
          550                        570                       590
CTTCTCAAATGAGACGTCATCTAAAGCACCCTGTAGAAAACACACAAATTGCAGTGTCTT
 F  S  N  E  T  S  S  K  A  P  C  R  K  H  T  N  C  S  V  F
          610                        630                       650
TGGTCTCCTGCTAACTCAGAAAGGAAATGCAACACACGACAACATATGTTCCGGAAACAG
 G  L  L  L  T  Q  K  G  N  A  T  H  D  N  I  C  S  G  N  S
          670                        690                       710
TGAATCAACTCAAAAATGTGGAATAGATGTTACCCTGTGTGAGGAGGCATTCTTCAGGTT
 E  S  T  Q  K  C  G  I  D  V  T  L  C  E  E  A  F  F  R  F
          730                        750                       770
TGCTGTTCCTACAAAGTTTACGCCTAACTGGCTTAGTGTCTTGGTAGACAATTTGCCTGG
 A  V  P  T  K  F  T  P  N  W  L  S  V  L  V  D  N  L  P  G
```

FIG.9D

```
          790                    810                    830
CACCAAAGTAAACGCAGAGAGTGTAGAGAGGATAAAACGGCAACACAGCTCACAAGAACA
  T   K   V   N   A   E   S   V   E   R   I   K   R   Q   H   S   S   Q   E   Q
              850                    870                    890
GACTTTCCAGCTGCTGAAGTTATGGAAACATCAAAACAAAGACCAAGATATAGTCAAGAA
  T   F   Q   L   L   K   L   W   K   H   Q   N   K   D   Q   D   I   V   K   K
          910                    930                    950
GATCATCCAAGATATTGACCTCTGTGAAAACAGCGTGCAGCGGCACATTGGACATGCTAA
  I   I   Q   D   I   D   L   C   E   N   S   V   Q   R   H   I   G   H   A   N
          970                    990                   1010
CCTCACCTTCGAGCAGCTTCGTAGCTTGATGGAAAGCTTACCGGGAAAGAAAGTGGGAGC
  L   T   F   E   Q   L   R   S   L   M   E   S   L   P   G   K   K   V   G   A
         1030                   1050                   1070
AGAAGACATTGAAAAAACAATAAAGGCATGCAAACCCAGTGACCAGATCCTGAAGCTGCT
  E   D   I   E   K   T   I   K   A   C   K   P   S   D   Q   I   L   K   L   L
         1090                   1110                   1130
CAGTTTGTGGCGAATAAAAAATGGCGACCAAGACACCTTGAAGGGCCTAATGCACGCACT
  S   L   W   R   I   K   N   G   D   Q   D   T   L   K   G   L   M   H   A   L
         1150                   1170                   1190
AAAGCACTCAAAGACGTACCACTTTCCCAAAACTGTCACTCAGAGTCTAAAGAAGACCAT
  K   H   S   K   T   Y   H   F   P   K   T   V   T   Q   S   L   K   K   T   I
         1210                   1230                   1250
CAGGTTCCTTCACAGCTTCACAATGTACAAATTGTATCAGAAGTTATTTTTAGAAATGAT
  R   F   L   H   S   F   T   M   Y   K   L   Y   Q   K   L   F   L   E   M   I
         1270                   1290                   1310
AGGTAACCAGGTCCAATCAGTAAAAATAAGCTGCTTATAACTGGAAATGGCCATTGAGCT
  G   N   Q   V   Q   S   V   K   I   S   C   L
         1330                   1350
GTTTCCTCACAATTGGCGAGATCCCATGGATGATAA
```

FIG.9E

```
                                                            50
muosteo.frg  MNKWLCCALLVLLDIIEWTTQETLPPKYLHYDPETGHQLLCDKCAPGTYL
ratosteo.frg MNKWLCCALLVLLDIIEWTTQETFPPKYLHYDPETGRQLLCDKCAPGTYL
huosteo.frg  MNKLLCCALVFLDISLIKWTTQETFPPKYLHYDETSHQLLCDKCPPGTYL

                                                            100
muosteo.frg  KQHCTVRRKTLCVPCPDHSYTDSWHTSDECVYCSPVCKELQSVKQECNRT
ratosteo.frg KQHCTVRRKTLCVPCPDYSYTDSWHTSDECVYCSPVCKELQTVKQECNRT
huosteo.frg  KQHCTAKWKTVCAPCPDHYYTDSWHTSDECLYCSPVCKELQYVKQECNRT

                                                            150
muosteo.frg  HNRVCECEEGRYLEIEFCLKHRSCPPGSGVVQAGTPERNTVCKKCPDGFF
ratosteo.frg HNRVCECEEGRYLEFCHKHRSCPPGLGVLQQAGTPERNTVCKRCPDGFF
huosteo.frg  HNRVCECKEGRYLEIEFCLKHRSCPPGFGVVQAGTPERNTVCKRCPDGFE

                                                            200
muosteo.frg  SGETSSKAPCIKHTNCSTFGLLIQKGNATHDNVCSGNREATQKCGIDVT
ratosteo.frg SGETSSKAPCRKHTNGSLGLLIQKGNATHDNVCSGNREATQKCGIQVT
huosteo.frg  SNETSSKAPCRKHTNCSVFGLLTQKGNATHDNICSGNSESTQKCGIDVT
```

FIG.9F

```
muosteo.frg   LCEEAFFRFAVPTKIIPNWLSVLVDSLPGTKVNAESVERIKRRHSSQEQT   250
ratosteo.frg  LCEEAFFRFAVPTKIIPNWLSVLVDSLPGTKVNAESVERIKRRHSSQEQT   250
huosteo.frg   LCEEAFFRFAVPTKFTPNWLSVLVDNLPGTKVNAESVERIKRQHSSQEQT   250

muosteo.frg   FQLLKLWKHQNRDQEMVKKIIQDIDLCESSVQRHLGHSNLTTEQLLALME   300
ratosteo.frg  FQLLKLWKHQNRDQEMVKKIIQDIDLCESSVQRHTGHANLTTEQLRILME   300
huosteo.frg   FQLLKLWKHQNKDQDIVKKIIQDIDLCENSVQRHIGHANLTFEQLRSLME   300

muosteo.frg   SLPGKKISPEEIERTRKTCKISSEQLKLLSLWRIKNGDQDTLKGLMHYALK  350
ratosteo.frg  SLPGKKISPDEIERTRKTCKPSEQLKLLSLWRIKNGDQDTLKGLMYALK   350
huosteo.frg   SLPGKKVGAEDIEKTIKACKPSDQTLKLLSLWRIKNGDQDTLKGLMHALK   350

muosteo.frg   HLKTSHFPKTVTHSLRKTMRFLHSFTHYRLYQKLFLEMIGNQVQSVKISC   400
ratosteo.frg  HLKAYHFPKTVTHSLRKTIRFLHSFTHYRLYQKLFLEMIGNQVQSVKISC   400
huosteo.frg   HSKTYHFPKTVTQSLRKTIRFLHSFTMYRLYQKLFLEMIGNQVQSVKISC   400

muosteo.frg   L   401
ratosteo.frg  L   401
huosteo.frg   L   401
```

FIG.10

```
ltnrr   CPQ-GKYIHPQNNSICCTKCHKGTYLYNDCPGPGQDTDCRECESGSFTAS   49
humoste PPKYLHYDEETSHQLCDKCPPGTYLKQHCTAK-WKTVCAPCPDHYTDS     49

ltnrr   ENHLRHCLSCS-KCRKEMGQVEISSCTVDRDTVCGCRKNQYRHYWSENLF   98
humoste WHTSDECLYCSPVCKELQYVK-QECNRTHNRVCECKEGRYLEI--E-F      93

ltnrr   QCFNCSLCLNG-TVHLSCQEKQNTVCT-CHAGFFLRE---NECVSC        139
humoste CKHRSCPPGFGVVQAGTPERNTVCKRCPDGFFSNETSSKAPCRKH          139
```

FIG.11

FIG.12A

DOMAIN 1
22- ETFPPKYLHYDEETSHQLLCDKCPPGTYLKQHCTAKWKTVCAP-64 HUMAN
22- ETLPPKYLHYDPETGHQLLCDKCAPGTYLKQHCTVRRKTLCVP-64 MOUSE
SS1   SS2   SS3

DOMAIN 2
65- CPDHYYTDSWHTSDECLYCSPVCKELQYVKQECNRTHNRVC-105
65- CPDHSYTDSWHTSDECVYCSPVCKELQSVKQECNRTHNRVC-105
SS1   SS2   SS3

DOMAIN 3
106-ECKEGRYLEIEFCLKHRSCPPGFGVVQAGTPERNTVC-142
106-ECEEGRYLEIEFCLKHRSCPPGSGVVQAGTPERNTVC-142
SS1   SS3

DOMAIN 4
143-KRCPDGFPSNETSSKAPCRKHTNCSVFGLLLTQKGNATHDNICSGNSESTQK-194
143-KKCPDGFPSGETSSKAPCIKHTNCSTPGLLLIQKGNATHDNVCSGNREATQK-194
SS1   SS3

N TERMINAL

FIG.12B

```
195-[C]GIDVTL[C]EEAPFRFAVPTKFTPNWLSVLVDNLPGTKVNAESVERIKRQHSS-246
195-[C]GIDVTL[C]EEAPFRFAVPTKIIPNWLSVLVDSLPGTKVNAESVERIKRRHSS-246

247-QEQTFQLLKLWKHQNKDQDIVKIIQDIDL[C]ENSVQRHIGHANLTPEQLRSL-298
247-QEQTFQLLKLWKHQNRDQEMVKIIQDIDL[C]ESSVQRHLGHSNLTTEQLLAL-298

299-MESLPGKKVGAEDIEKTIKA[C]KPSDQILKLLSLWRIKNGDQDTLKGLMHALK-350
299-MESLPGKKISPEEIERTRKT[C]KSSEQLLKLLSLWRIKNGDQDTLKGLMYALK-350

351-HSKTYHFPKTVTQSLKKTIRPLHSFTMYKLYQKLFLEMIGNQVQSVKIS[C]L-401
351-HLKTSHFPKTVTHSLRKTMRFLHSFTMYRLYQKLFLEMIGNQVQSVKIS[C]L-401
```
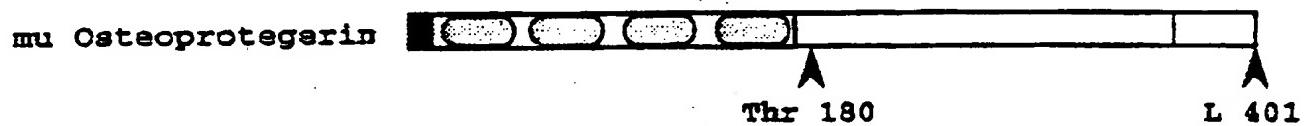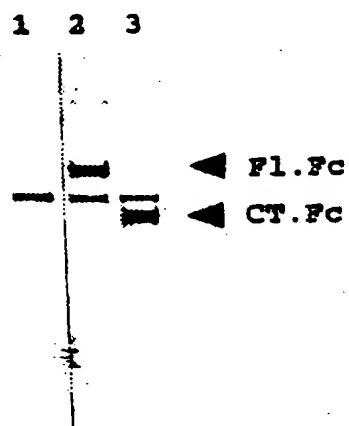
C
TERMINAL

FIG.13A

mu Osteoprotegerin

Thr 180   L 401

FIG.13B

1　2　3

◄ Fl.Fc

◄ CT.Fc

FIG.13C

1　2　3

◄ Fl.Fc

◄ CT.Fc

FIG.14A

FIG.14B

FIG.15

Reduced Non-Reduced

110 kd

55 kd
50 kd

FIG.16A

Cell Lysate   **Medium**

T₀ 0.5 1 2 4 6 12 hr   T₀ 0.5 1 2 4 6 12 hr

Non-Reducing

-100 K —   — dimer

-55 K =   — monomer

FIG.16B

T₀ 0.5 1 2 4 6 12 hr   T₀ 0.5 1 2 4 6 12 hr

Reducing

-55 K =   — monomer

FIG.17

FIG.18

Serum   **Liver**
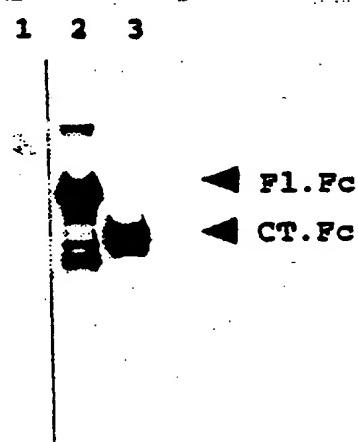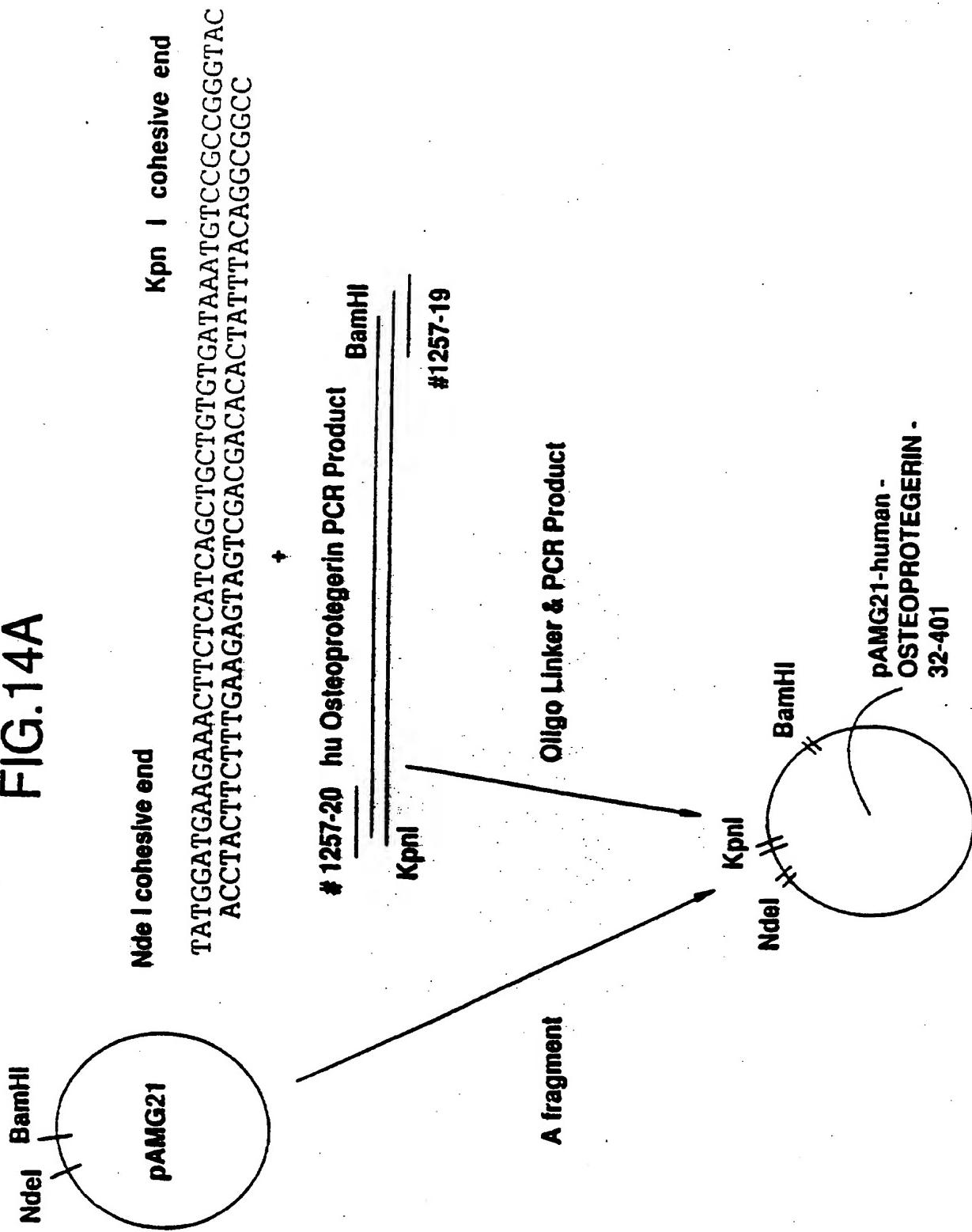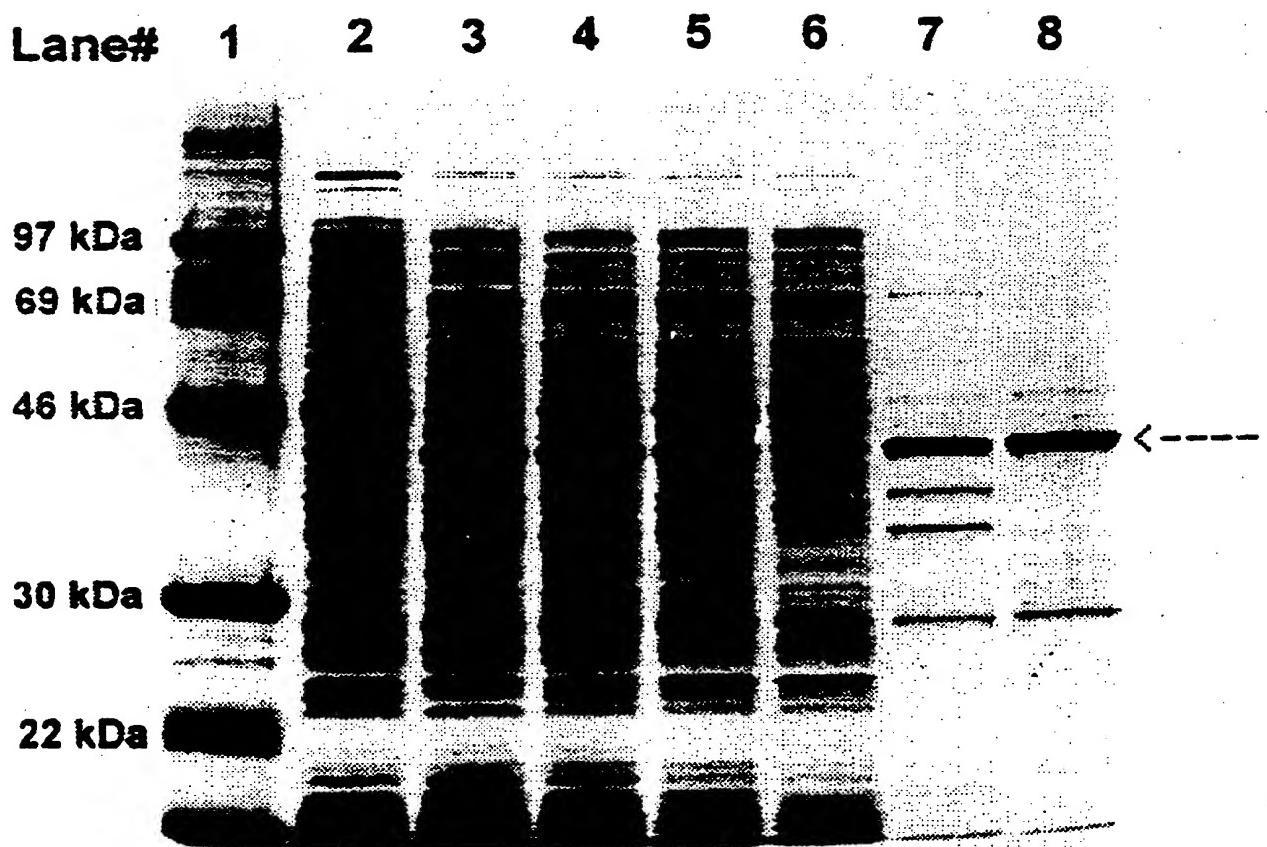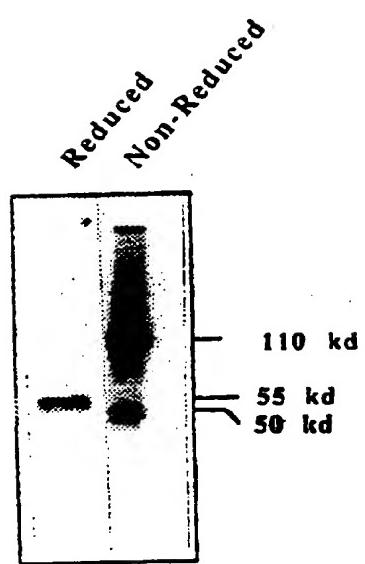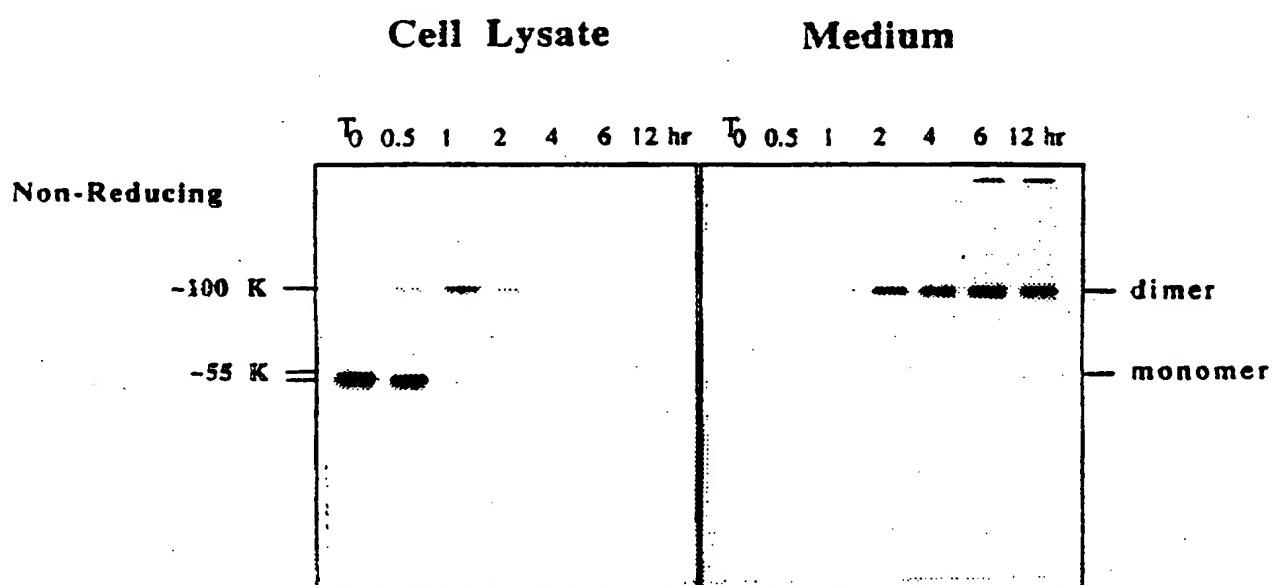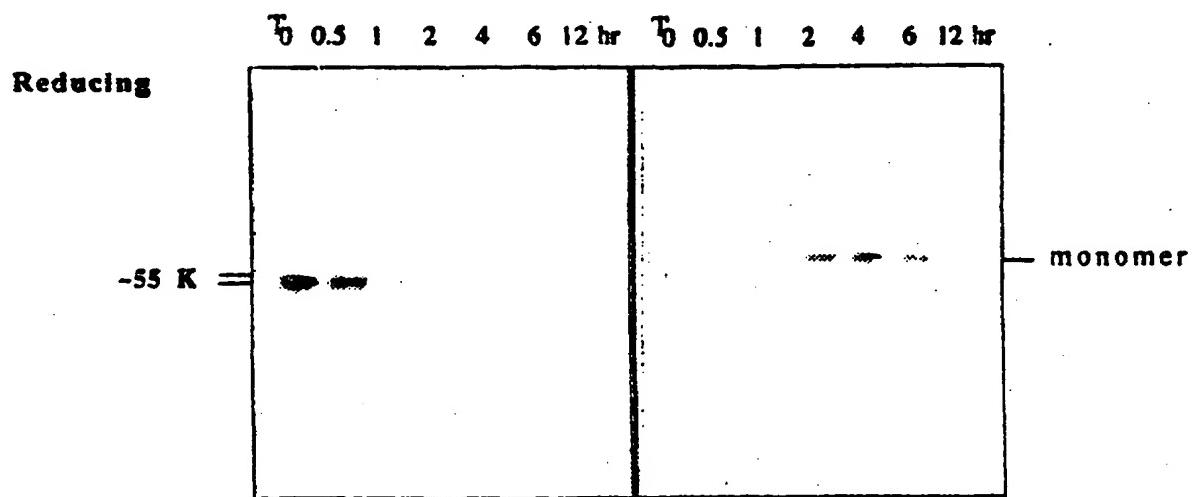
FIG.19B
OPG - 10 ng/ml
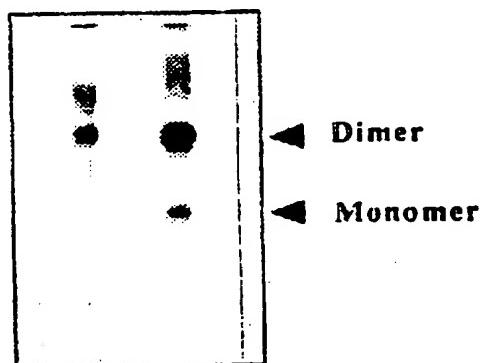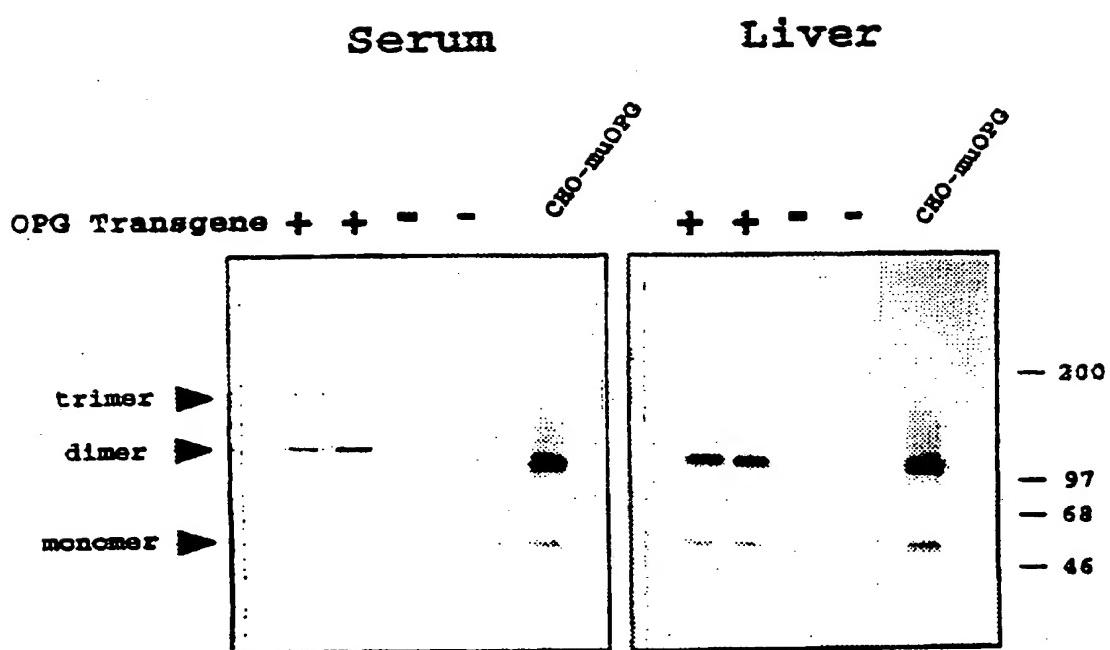
FIG.19D
OPG - 0.1 ng/ml

FIG.19A
OPG - 100 ng/ml
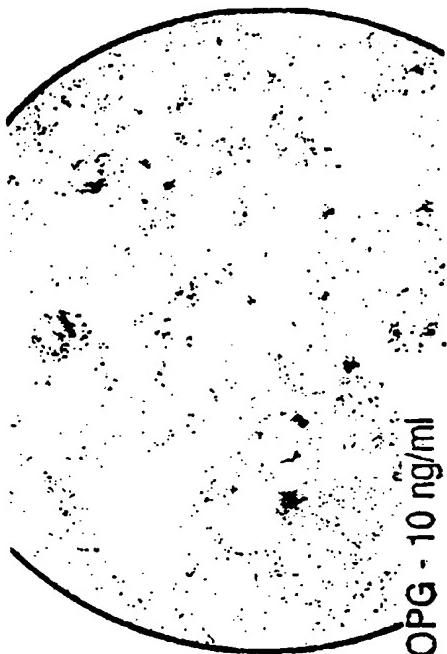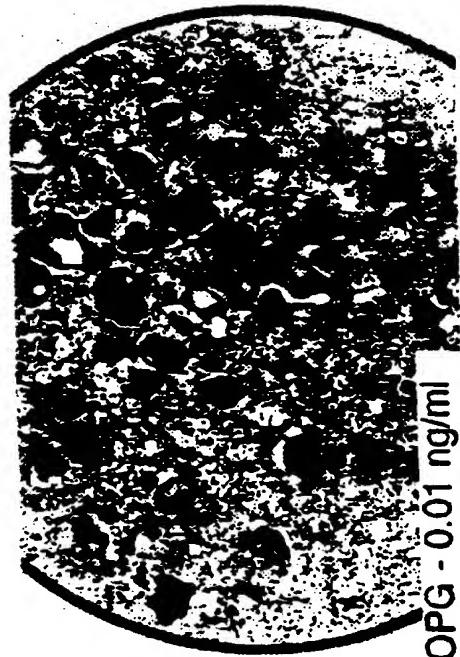
FIG.19C
OPG - 1 ng/ml

FIG.19F

OPG - 0.001 ng/ml

FIG.19E

OPG - 0.01 ng/ml

FIG.19G

Control

FIG.20

FIG.21

A405

| | OPG-CTL | OPG-OPG | CTL-OPG | CTL-CTL |
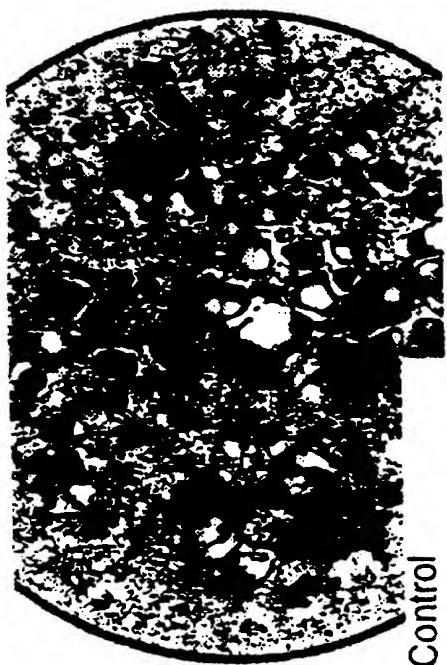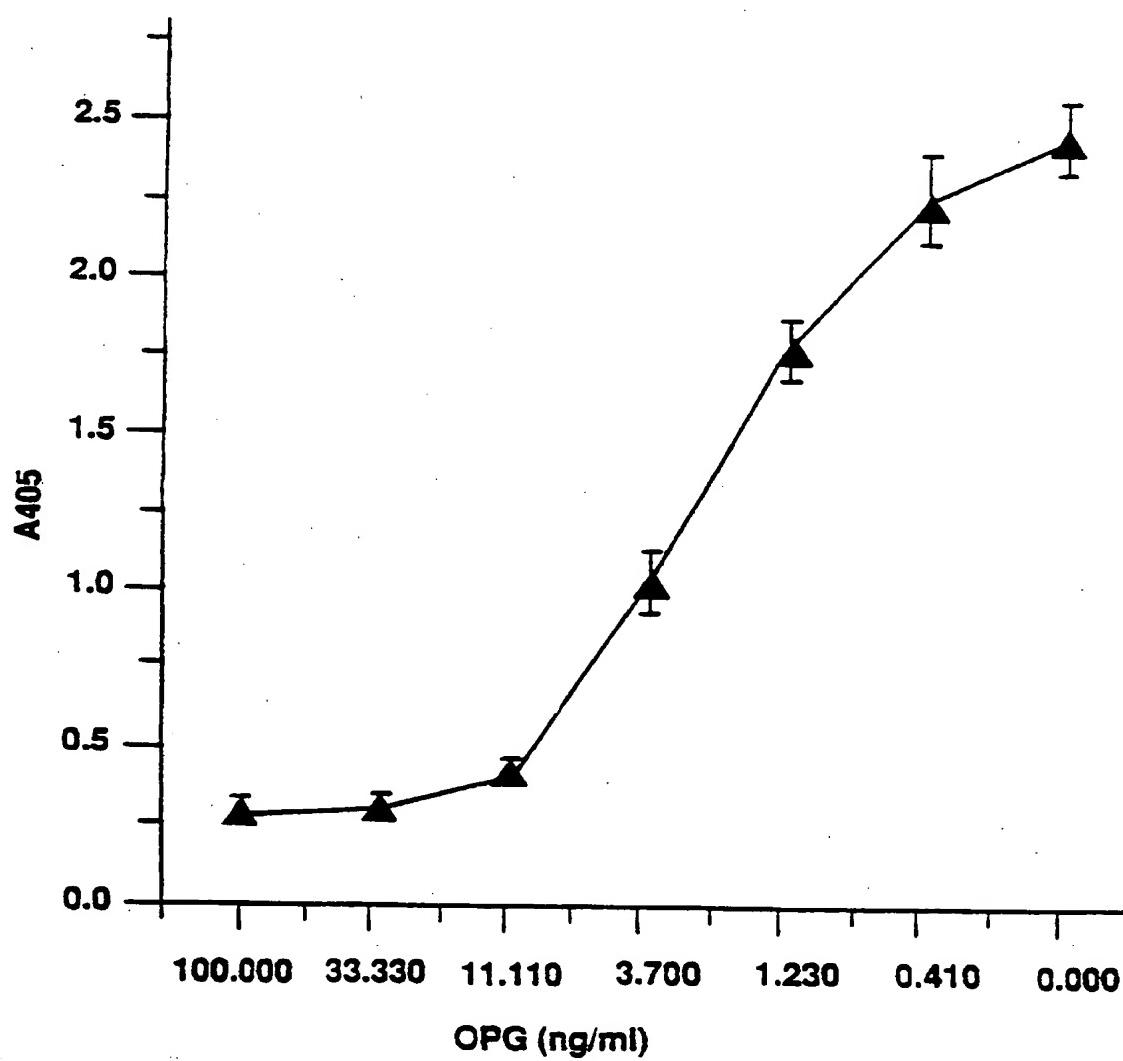
Legend
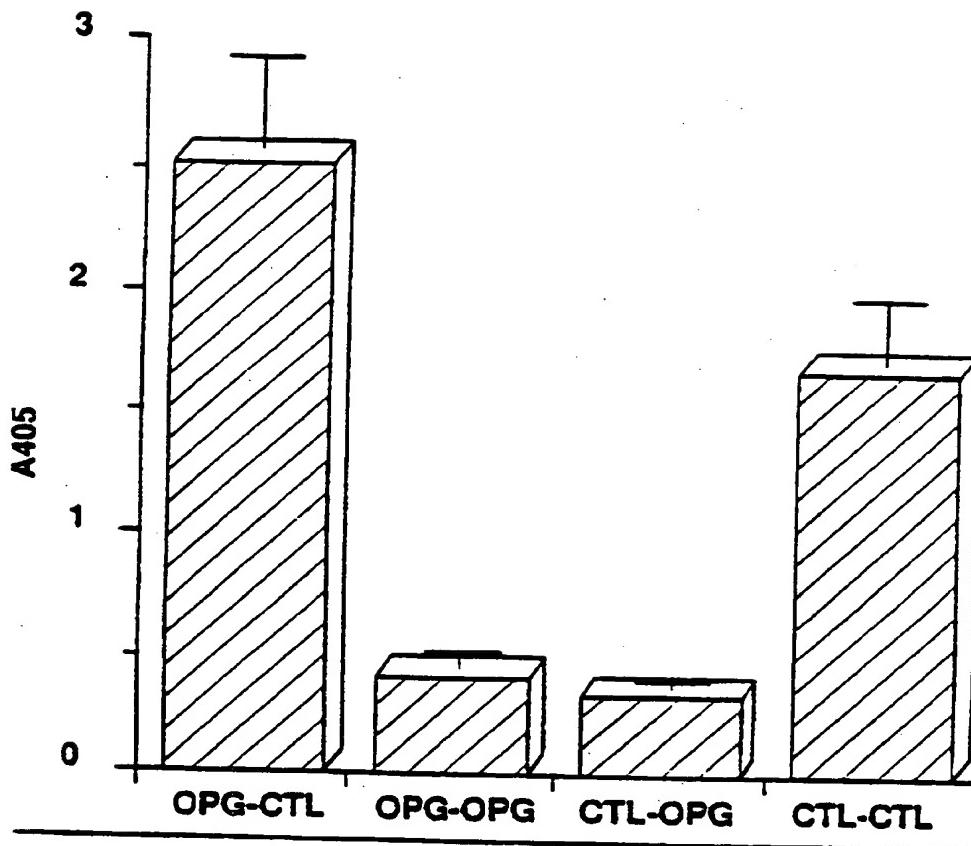
Growth	Intermediate	Terminal
Bone marrow cells CSF-1	PGE2 + CSF-1	ST2 cells 1,25 (OH)2 D3 Dexamethasone
4 days	2 days	8 - 10 days

Groups	OPG	OPG
CTL - CTL	– – –	– – –
OPG - CTL	100 ng/ml	– – –
OPG - OPG	– – –	100 ng/ml
OPG - OPG	100 ng/ml	100 ng/ml

FIG.22A
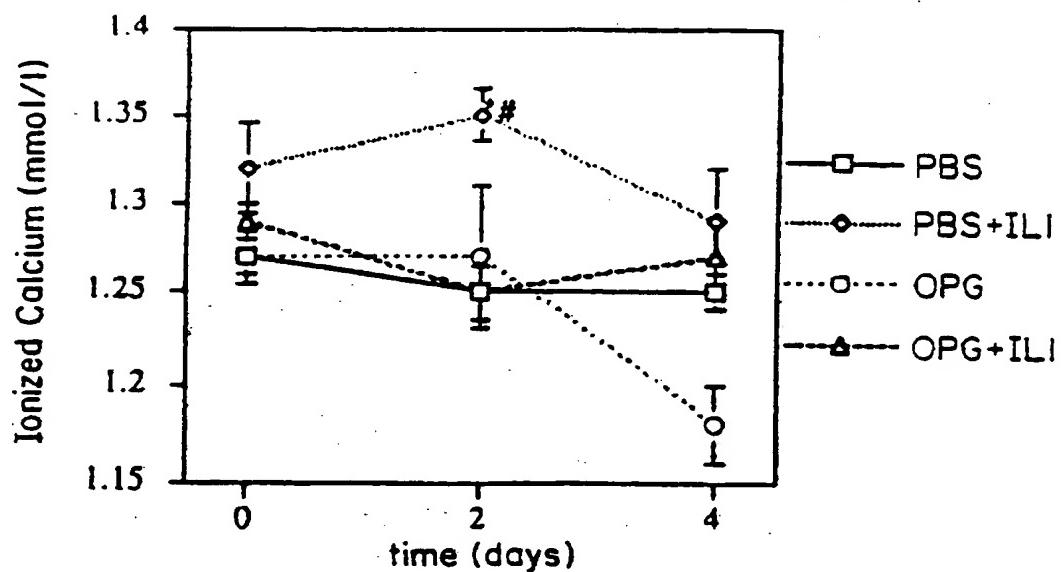
FIG.22B
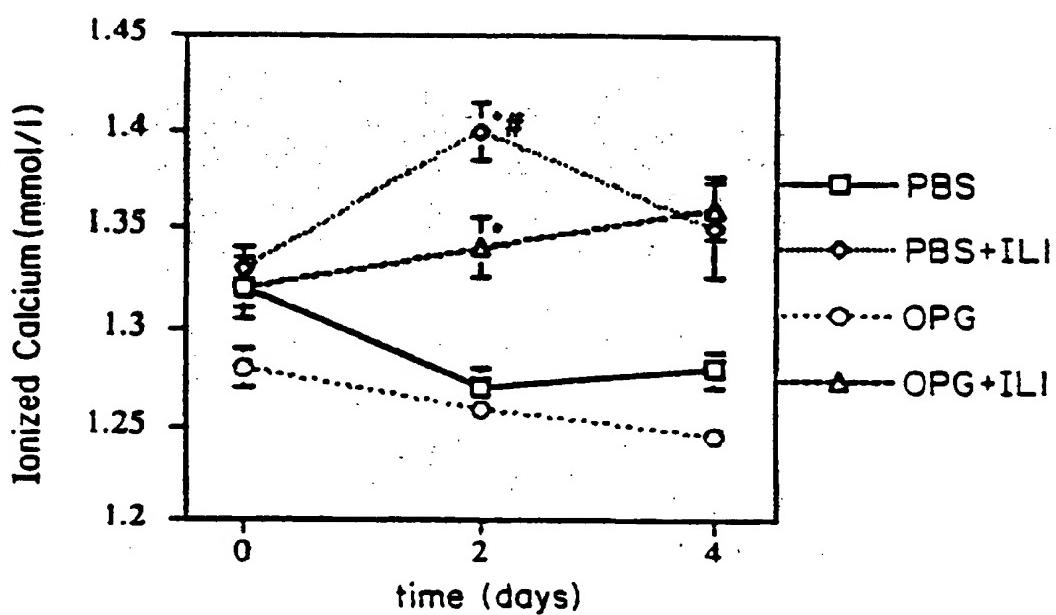
* Different to PBS, p < 0.05
# Different to OPG + IL1, p < 0.05

FIG.23A

PBS/PBS

FIG.23B

IL1/PBS

FIG.23C

PBS/OPG
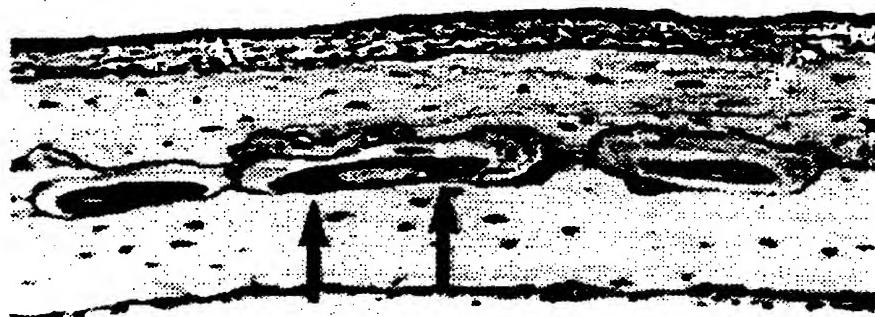
FIG.23D

IL1/OPG
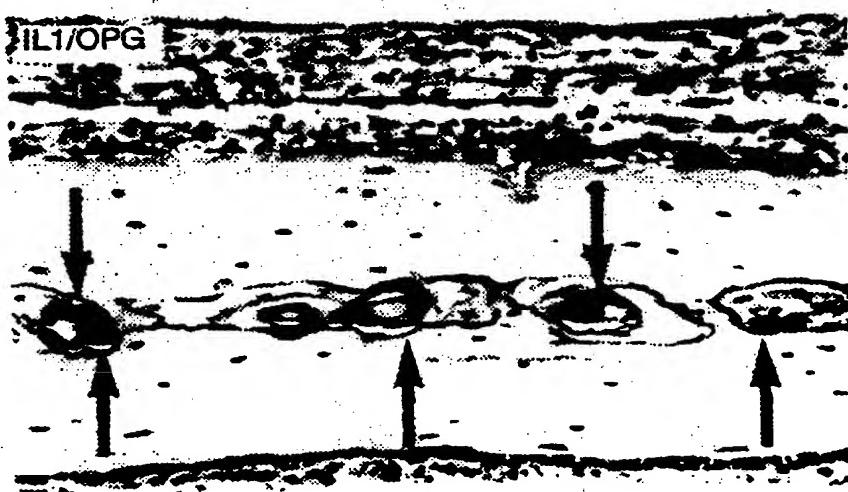
FIG.24B

FIG.24A
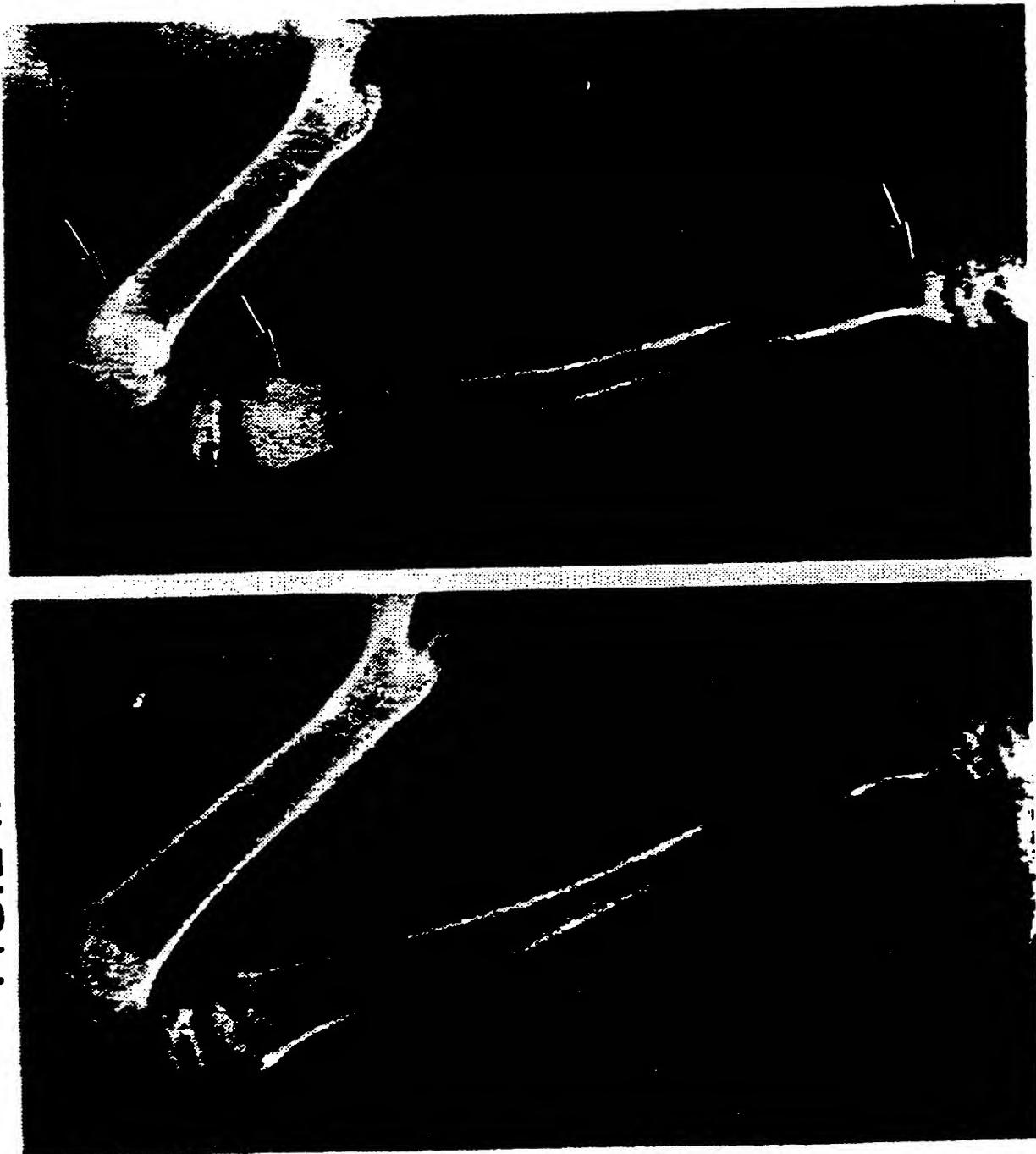
FIG.25
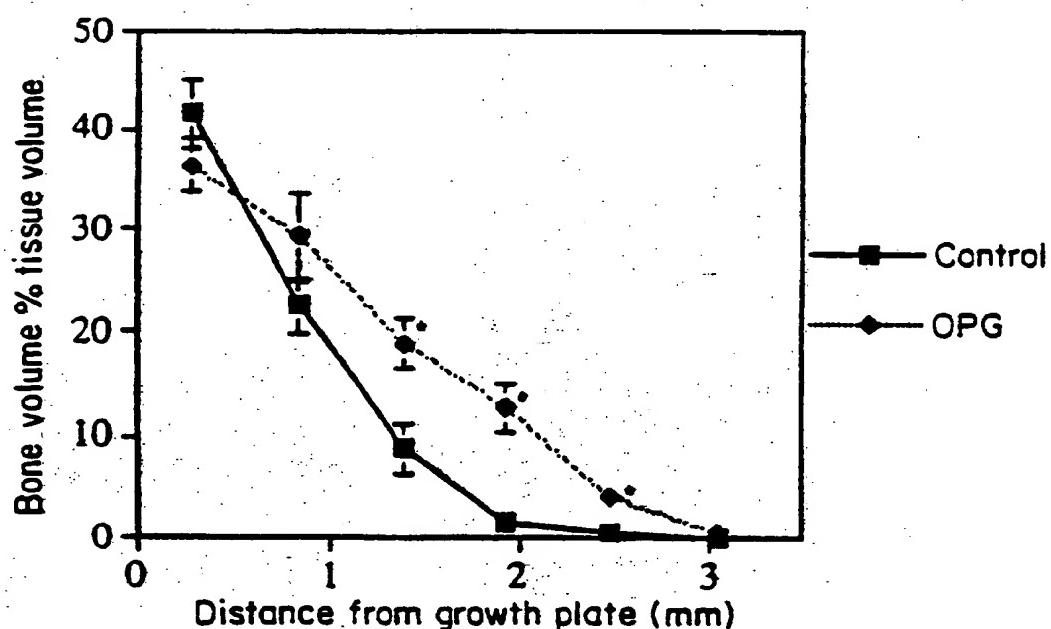
* Different to control p < 0.01

FIG.26A

FIG.26.B

FIG.27
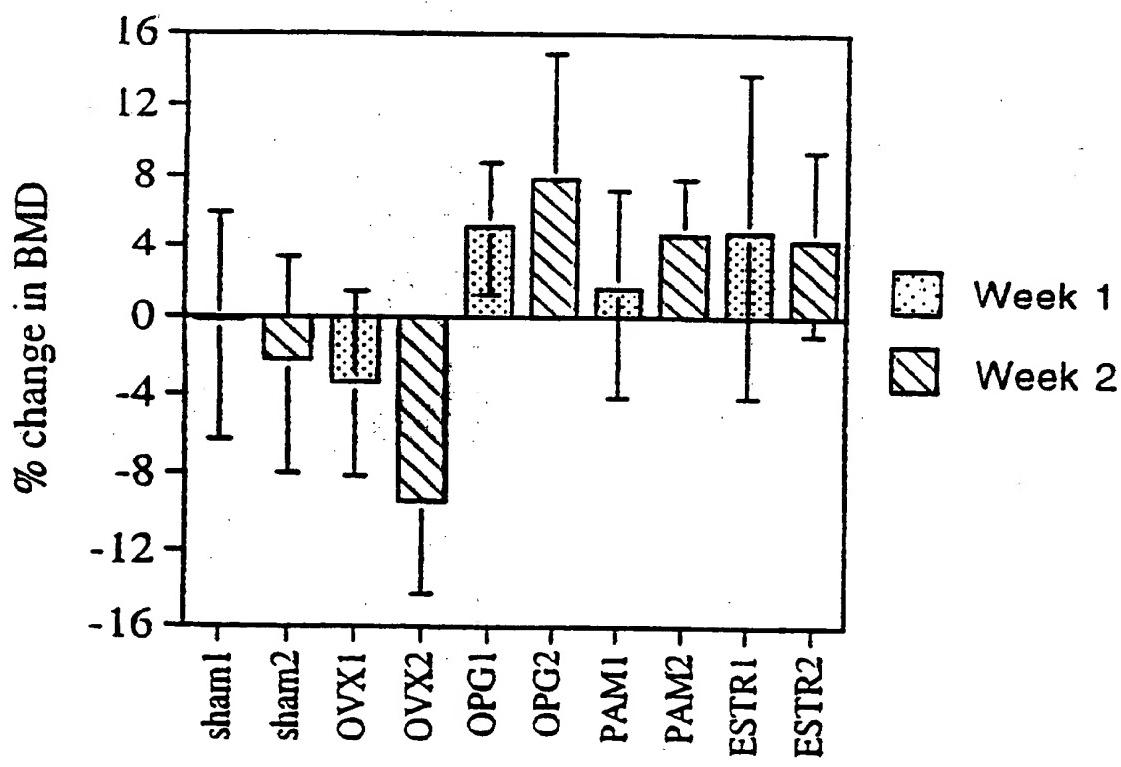
FIG.28
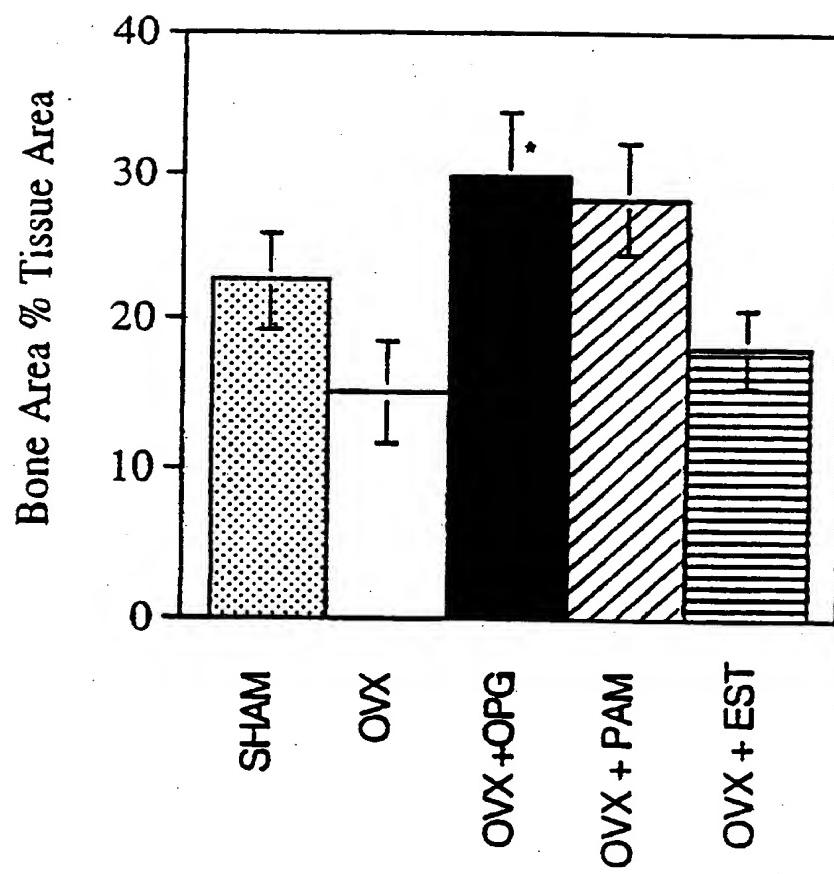
* Different to OVX p < 0.05

Inter... .al Application No

PC1/US 96/20621

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/12   C07K14/715   C12N5/10   A01K67/027   C07K19/00
        C12N15/62   C07K16/28   C07K1/107 C12Q1/68   G01N33/50
        G01N33/566 A61K38/17   A61K48/00 C12N1/21   //(C12N1/21,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   A01K   C12Q   G01N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CELL, vol. 76, 25 March 1994, pages 959-962, XP002029050 SMITH C.A. ET AL.: "The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death." cited in the application see the whole document ---	1-60
A	PROC.NATL.ACAD.SCI.USA, vol. 88, 1991, pages 2830-2834, XP002029051 LEWIS M. ET AL.: "Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific." see figure 1 ---	1-60

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 April 1997	16. 04. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Mandl, B

Form PCT/ISA/210 (second sheet) (July 1992)

Inte   mal Application No

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, vol. 252, 1991, pages 1651-1656, XP000645049 ADAMS M.D.: "Complementary DNA sequencing:expressed sequence tags and human genome project." cited in the application see the whole document ---	1-60
A	US 5 447 851 A (BEUTLER BRUCE A ET AL) 5 September 1995 see the whole document ---	36-38,57
A	US 4 179 337 A (DAVIS FRANK F ET AL) 18 December 1979 cited in the application see the whole document -----	28,29

☐ Further documents are listed in the continuation of box C.   ☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   43-45,49-53
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although these claims are directed to a method of treatment of
           (diagnostic method practised on) the human/animal body, the search
           has been carried out and based on the alleged effects of the com-
           pound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
        searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
        of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report
        covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
        restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                                                 [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5447851 A	05-09-95	NONE	
US 4179337 A	18-12-79	CA 1033673 A	27-06-78
		CH 616942 A	30-04-80
		DE 2433883 A	05-02-76
		FR 2313939 A	07-01-77
		GB 1469472 A	06-04-77
		JP 1152589 C	30-06-83
		JP 50042087 A	16-04-75
		JP 56023587 B	01-06-81
		NL 7409770 A	22-01-75
		SE 441753 B	04-11-85
		SE 7409366 A	21-01-75